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Relation of the micro-RNA content of unsorted cryopreserved sperm and the fertility of sperm after sex-sorting

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ABSTRACT

In this study, we hypothesised that the microRNA (miRNA) profile of cryopreserved unsorted (conventional) bovine sperm is related to bull fertility after artificial insemination (AI) with X-bearing sperm. Eighteen bulls were selected based on their annual 56-day non-return rate after AI with conventional (NRR_{conv}) and sex-sorted sperm (NRR_{ss}), and assigned to two equal groups: low- (LF) and high-fertility (HF). The LF and HF bulls had similar NRR_{conv} (69.09%±2.82%) but their NRR_{ss} was reduced by 22.33%±4.01% and 8.63%±1.71%, respectively. Small RNA libraries prepared from a pool of four ejaculates per bull were sequenced with an Illumina HiSeq 2500 system. The Spearman's rank correlation coefficient (r_s) was used to evaluate the relation between NRR values and miRNA expression levels; the value of the latter as predictor of NRR_{ss} was assessed with robust regression analysis. Out of 85 totally detected miRNAs, miR-34b-3p and miR-100-5p were the two most abundant. MiR-10a-5p and miR-9-5p were differentially expressed in LF and HF samples (false discovery rate<10%). MiR-9-5p, miR-34c, miR-423-5p, miR-449a, miR-5193-5p, miR-1246, miR-2483-5p, miR-92a, miR-21-5p were related to NRR_{ss} ($0.515 \leq |r_s| \leq 0.693$, $P<0.05$) but not to NRR_{conv} ($P>0.05$). MiR-34c, miR-7859, miR-342, miR-106b-5p and miR-92a showed the highest contribution to the prediction of NRR_{ss}. Concluding, bull fertility after AI with sex-sorted sperm appeared to be linked with the miRNA profile of sperm before sorting.

Key words: bull fertility; sex-sorted sperm; microRNA; small RNA-seq

Zusammenfassung

Es wurde überprüft, ob die Fertilität von kryokonserviertem, gesextem Bullensperma anhand der micro-RNA (miRNA) in konventionell hergestelltem Tiefgefriersperma beurteilt werden kann. Dazu wurde Sperma von 18 Bullen ausgewählt, bei denen die Fertilität anhand der Non-Return Rate bekannt war (NRR_{conv} u. NRR_{ss} für konventionelles bzw. für gesextes Sperma). Die Bullen wiesen ähnliche NRR_{conv} (69.09%±2.82%) auf. Bei 9 Bullen war die NRR_{ss} deutlich gegenüber der NRR_{conv} verringert (Δ = 22.33%±4.01%; Low Fertility = LF), während bei den anderen 9 Bullen die NRR_{ss} im Vergleich zur NRR_{conv} nur moderat reduziert war (Δ = 8.63%±1.71%; High Fertility = HF)). Für die Sequenzierung der miRNAs wurden von jedem Bullen Proben je 4 Ejakulate gepoolt. Es wurden 85 miRNAs identifiziert, wobei die miR-34b-3p und miR-100-5p am stärksten und die miR-10a-5p und miR-9-5p zwischen den Ejalukaten von HF- und LF-Bullen unterschiedlich stark exprimiert waren ($P<0.05$). Die miR-9-5p, miR-34c, miR-423-5p, miR-449a, miR-5193-5p, miR-1246, miR-2483-5p, miR-92a und miR-21-5p korrelierten gut mit der NRR_{ss} ($0.515 \leq |r_s| \leq 0.693$, $P<0.05$; r_s = Spearman'scher Rangkorrelationskoeffizient), aber nicht mit der NRR_{conv} ($P>0.05$). Die miR-34c, miR-7859, miR-342, miR-106b-5p und miR-92a waren am aussagekräftigsten zur Prognose der NRR_{ss} (robuste Regressionsanalyse). Zusammenfassend lieferten die miRNAs konventionell hergestellten Tiefgefrierspermas wertvolle Hinweise auf die Fruchtbarkeit gesexten Bullenspermas.

Schlüsselwörter: Bullenfruchtbarkeit; gesextes Sperma; microRNA; small RNA-Seq

Abbreviations

%DFI	Percentage of sperm with high DNA fragmentation index
AI	Artificial insemination
AM	Acetoxymethyl
AO	Acridine orange
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
BP	Band-pass
Ca^{2+}	Calcium
CASA	Computer-assisted sperm analysis
CI	Confidence intervals
cpm	Count per million
$C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$	Sperm with a high intracellular esterase activity, a high mitochondrial membrane potential, a low intracellular calcium level and intact plasma and acrosome membrane
DAVID	Database for annotation, visualization and integrated discovery
DE	Differentially expressed
DEPC	Diethyl pyrocarbonate
DFI	DNA fragmentation index
$DiIC_1(5)$	1,1',3,3,3',3'-Hexamethylindodicarbocyanine iodide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
FGCZ	Functional Genomic Center Zurich
GO	Gene ontology

HF	High fertility
IGF2R	Insulin-like growth factor 2 receptor
KCl	Potassium chloride
LF	Low fertility
M_{conv}	Robust regression line for the prediction of the non-return rate of conventional sperm
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
M_{ss}	Robust regression line for the prediction of the non-return rate of sex-sorted sperm
NaOAc	Sodium Acetate
NCBI	National Center for Biotechnology Information
NRR	Non-Return Rate
NRR_{conv}	Non-return rate for conventional sperm
NRR_{ss}	Non-return rate for sex-sorted sperm
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE-PNA	Phycoerythrin-conjugated agglutinin of *Arachis hypogea*
PI	Propidium Iodide
piRNA	Piwi-interacting RNA
PMAI	Sperm with an intact plasma membrane and acrosome
rcf	Relative centrifugal force
r_s	Spearman's rank correlation coefficient
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
sncRNA	Small non-coding RNA
TAE	Tris-acetate-EDTA
TEMED	N,N,N,N'-tetramethylenediamine
TNE	Tris-NaCl-EDTA
VIF	Variance inflation factor
Δ_{NRR}	Relative change of the non-return rate

1. INTRODUCTION

Manipulating the calf sex ratio can be a powerful tool for increasing the profitability and for accelerating the genetic gain in dairy and beef cattle farming (Seidel, 2014; Ettema and Østergaard, 2015; Osada et al., 2019). Thus, it is not a surprise that the use of sex-sorted sperm in bovine assisted reproduction has steadily increased in the last years (Hutchison and Bickhart, 2016; Heuer et al., 2017). Although alternative methodologies have been described (Rath et al., 2013; Asma-ul-Husna et al., 2017; Umehara et al., 2019), the separation of X- and Y-bearing spermatozoa by means of flow cytometry after Hoechst 33342 labeling is still the technique of choice applied in most sorting facilities, mainly due to its high accuracy, repeatability and suitability for commercial application (Vishwanath and Moreno, 2018). Nevertheless, several research groups had already reported that inseminating dairy heifers with a dose of 1 to 2 million frozen-thawed X-bearing sperm resulted in conception rates not higher than 70-90% of these achieved with unsorted sperm (henceforward mentioned as "conventional" in the text; (Seidel and Schenk, 2008; DeJarnette et al., 2009; Schenk et al., 2009; Chebel et al., 2010; Norman et al., 2010; Butler et al., 2014). Consequently, along with the higher price of sex-sorted products, a variable loss in bull fertility appeared to be the major cost of artificial insemination (AI) with sex-sorted sperm (Seidel, 2003; Olynk and Wolf, 2007) and, thus, a considerable drawback to the global expansion of its use.

Recent advancements in sorting technology in combination with an almost two-fold increase of the usual AI dose (i.e. 2.1 million sex-sorted sperm per dose) are expected to address the fertility problem both in heifers and cows, resulting in non-return rate (NRR) values of approximately 90% of those obtained after AI with conventional sperm (Lenz et al., 2017; Thomas et al., 2017, 2019). Nonetheless, the production of sex-sorted sperm remains an expensive procedure and processing ejaculates of sires that do not perform optimally after sex-sorting costs a considerable amount of resources. Post-thaw quality characteristics of sex-sorted sperm can be of some predictive value for its fertilizing potential after AI (Holden et al., 2017); however, this information is available only at late stages of the production process, when sire and ejaculate selection, semen logistics, sperm sorting and cryopreservation, all time-consuming and costly procedures, have already taken place. Not surprisingly, the NRR for conventional semen (NRR_{conv}) has not been proven a reliable indicator of the NRR for sex-sorted semen (NRR_{ss}) either, even when equal doses of both semen types were used for the generation of NRR data (Bodmer et al., 2005; Dejarnette et al., 2011). Indeed, a large study on dairy bulls used for the production of both conventional and sex-sorted sperm in the U.S.A. demonstrated that sire fertility rankings significantly differ between the two semen types (Norman et al., 2011). Several studies have shown that the fertilizing potential of sperm after sorting largely varies between bulls when used either for field AI (DeJarnette et al., 2008; Schenk et al., 2009; Dejarnette et al., 2010a, 2011; Thomas et al., 2019) or for *in vitro* embryo production (Xu et al., 2006; Blondin et al., 2009; Inaba et al., 2016). It is well known that NRR values respond to increasing sperm doses in a bull-dependent manner; this response pattern is linked to the level of non-compensable defects present in sperm and has been documented for both conventional (Den Daas et al., 1998; Dejarnette et al., 2010b) and sex-sorted sperm (DeJarnette et al., 2008). There is also indication that sperm tolerance to mechanical stress (i.e. sorting pressure) and prolonged storage prior to sorting varies between individuals (Schenk et al., 2009). Interestingly, sex-sorting affects sperm molecular mechanisms in a bull-dependent manner too. In a split-ejaculate experiment, Carvalho et al. (2012) observed that the effects of the sorting procedure on the methylation profile of the *IGF2R* gene of Y-bearing sperm differed significantly between bulls. Thus, a better understanding of bull-specific factors related to the functional status and molecular biology of sperm cells after sorting would substantially

contribute to fertility prognosis of sex-sorted sperm (Seidel, 2012; Vishwanath and Moreno, 2018).

Studies about the impact of sex-sorting on the molecular features of sperm and the respective consequences for male fertility are rather scarce. It has been shown that both sex-sorting and cryopreservation induce epigenetic changes to sperm, particularly related to their gene methylation pattern (Carvalho et al., 2012) and transcriptome profile (Dai et al., 2019; Zeng et al., 2014). In the same direction, Morton et al. (2007) described differences in the relative transcript abundance of developmentally relevant genes between day-7 bovine embryos that were *in vitro* produced using conventional and sex-sorted sperm. Similar findings have also been reported in other ruminant species (Beilby et al., 2011). The authors attributed the differential expression of these genes to alterations of sperm molecular characteristics after sex-sorting; however, the nature of these alterations was not further investigated (Morton et al., 2007).

Among other RNA molecules, small non-coding RNAs (sncRNA), i.e. transcripts with length of less than 200 nucleotides that do not serve as template for protein synthesis, have rapidly attracted interest of researchers in the field of animal reproduction in the last decade, mainly due to their potential use as fertility biomarkers (Reza et al., 2019). Bovine spermatozoa are equipped with a wide array of sncRNAs including microRNAs (miRNA) and Piwi-interacting RNAs (piRNA) (Robertson et al., 2008; Govindaraju et al., 2012; Du et al., 2014; Sellem et al., 2020). Several studies have focused on the relation between sperm miRNA profile and bull fertility (Govindaraju et al., 2012; Malama et al., 2018; Sellem et al., 2018; Menezes et al., 2020). Although, mature sperm are considered transcriptionally silent, their miRNA content shows a dynamic response to stressful procedures, like cryopreservation (Dai et al., 2019; Zhang et al., 2017) and induction of capacitation (Li et al., 2018). Indeed, the transcriptome profile of porcine spermatozoa has recently been suggested as an indicator of their freezability and, thus, their ability to tolerate stress related to semen processing (Fraser et al., 2020). Moreover, it is known that miRNA genes located on the X chromosome are capable of escaping the meiotic sex chromosome inactivation, i.e. the transcriptional silencing of the unsynapsed X- and Y-chromosomal region at the onset of pachynema in mammalian male germ cells (Sosa et al., 2015). X-linked miRNAs remain active even until the onset of spermiogenesis and serve as post-transcriptional regulators of spermatogenesis at the late meiotic and post meiotic phases (Turner, 2007). Despite the increasing evidence about the dynamics of miRNAs in mature sperm and their role in the inactivation/activation cycle of the X and Y chromosome during sperm cell development, their profile in sperm lined up for sex-sorting has not been adequately studied yet.

In the present study, we tested the hypothesis that the miRNA profile of conventional semen is related to the fertility outcome of AI with X-bearing sperm. For this purpose, we assessed the sperm functional status and miRNA profile in conventional AI doses produced from proven sires with diverse fertility after sorting.

2. MATERIAL AND METHODS

2.1. Biological material

Bull and sperm sample selection. For this study, 18 bulls were selected from a pool of 50 sires housed in a single AI station under uniform management and feeding conditions. All bulls were in parallel used for the production of conventional and sex-sorted (X-bearing sperm at 90% purity) cryopreserved sperm doses. Fertility of sires after AI with conventional and sex-sorted semen was systematically recorded in form of an annual % 56-day NRR after a minimum of 500 first services (NRR_{conv} and NRR_{ss}, respectively). For each bull the relative change of the NRR (Δ_{NRR}) after AI with conventional and sex-sorted semen was computed according to the following formula:

$$\Delta_{NRR}\ (\%) = 100 \times [(NRR_{conv} - NRR_{ss}) / NRR_{conv}]$$

Eighteen bulls with Δ_{NRR} values lying at the extremes of the Δ_{NRR} distribution of the 50 sires, i.e. outside the range of mean Δ_{NRR} ± SD, were selected and assigned in two equal groups: nine bulls (five Holstein-Friesian, one Red Holstein, two Swiss Fleckvieh and one Simmental bull) with low fertility (LF; n_{LF}=9), and nine bulls (three Holstein-Friesian, two Red Holstein, three Brown Swiss and one Limousin bull) with high fertility (HF; n_{HF}=9) after sex-sorting. The mean values±SD of fertility data (number of first services with conventional and sex-sorted sperm, NRR_{conv}, NRR_{ss}, Δ_{NRR}) in relation to fertility group are presented in Table 1. Bulls of both groups showed similar NRR_{conv} values (69.57%±3.33% and 68.61%±2.09% for LF and HF bulls, respectively); however, the NRR values of LF bulls were reduced by 22.33% after AI with sex-sorted sperm against a reduction of only 8.63% observed for the HF bulls (Table 1).

Semen collection and processing. The cryopreserved sperm samples examined in this study originated from the regular semen collection schedule of the AI center. Semen was collected by using a pre-warmed (38 °C) artificial vagina after the bulls mounted on a dummy bull or cow. Ejaculates were evaluated immediately after ejaculation in terms of ejaculate volume, sperm concentration, progressive motility and morphology using a phase contrast microscopy with 100× magnification. Only ejaculates fulfilling the criteria of volume ≥2 mL, sperm concentration ≥600 × 10^6 sperm/mL and progressive motility ≥70% were further processed and cryopreserved.

Semen was extended with a Triladyl®-egg yolk extender [250 g Triladyl® (Minitube GmbH, Tiefenbach, Germany), 750 mL distilled water, 250 mL egg yolk] to a final concentration of 65 × 10^6 sperm/mL and packaged in 0.25 mL French straws (IMV Technologies; L'Aigle, France) using a fully automatic straw filling and sealing machine (IS4, IMV Technologies, Aîgle, France). Semen was then cooled to 4 °C for 24 h before freezing by means of a computer-assisted freezing chamber (Digitcool 5300 3T, IMV Technologies, Aîgle, France) with a temperature decrease rate of 5 °C/min to -10 °C, then 40 °C/min to -110 °C and finally 20 °C/min to -140 °C. Right after, straws were transferred and stored in liquid nitrogen (-196 °C).

In total, 72 unsorted and cryopreserved ejaculates (four conventional semen batches per bull) were used as input for laboratory semen examination and analysis of their small non-coding RNA profile.

Table 1. Fertility data of 18 bulls in relation to the reproductive performance of the bull after artificial insemination (AI) with sex-sorted semen (low fertility, LF; high fertility, HF). The 56-day non-return rate after AI with cryopreserved unsorted (conventional) and sex-sorted sperm (NRR_{conv} and NRR_{ss}, respectively) of each sire was recorded on annual basis; the relative change of NRR after AI with sex-sorted against conventional semen (Δ_{NRR}) was computed according to the formula: Δ_{NRR} (%) = 100 × [($NRR_{conv} - NRR_{ss}$) / NRR_{conv}].

	LF				HF				Total			
	n	Mean±SD	Min	Max	n	Mean±SD	Min	Max	n	Mean±SD	Min	Max
First AI with conventional sperm	9	7232±4466	1931	13656	9	11549±16817	613	57705	18	9391±12491	613	57705
First AI with sex-sorted sperm	9	1208±357	741	1836	9	2674±2805	520	9863	18	1941±2130	520	9863
NRR_{conv} (%)	9	69.57±3.33	64.11	73.85	9	68.61±2.09	64.97	72.58	18	69.09±2.82	64.11	73.85
NRR_{ss} (%)	9	54.10±4.00	45.58	59.81	9	62.69±2.74	58.13	68.10	18	58.40±5.77	45.58	68.10
Δ_{NRR} (%)	9	22.33±4.01	31.44	18.84	9	8.63±1.71	10.53	5.25	18	15.48±7.50	31.44	5.25

2.2. Laboratory sperm analysis

2.2.1 Preparation of semen prior to analysis

Four straws from each batch were thawed in a water bath (38 °C, 30 sec) and pooled in a pre-warmed (38 °C) 1.5-mL laboratory tube. Pooled samples were further assessed with computer-assisted sperm analysis and flow cytometry immediately after thawing (0h) at 38 °C in 5% CO_2 humidified atmosphere. Materials and buffers or staining solutions contacting with sperm were pre-warmed at 38 °C.

2.2.2. Computer-assisted sperm analysis (CASA)

Sperm motility and kinematics were assessed using an IVOS II CASA system driven by the software version 1.10.1 (Hamilton Thorne Inc., Beverly, U.S.A.). A pre-warmed (38 °C) 20 μm-deep 4-chamber Leja slide (IMV Technologies; L'Aigle, France) was filled with 6 μL of semen, and a minimum of 1,000 cells were analyzed in at least eight randomly selected fields with 30 frames acquired per field at a frame rate of 60 Hz. Sperm with straightness ≥70% and average path velocity ≥50 μm/s were considered progressively motile. In each sample, the percentage of progressively motile sperm (progressive motility) was recorded.

2.2.3. Flow cytometric analysis of sperm

Chemicals and Reagents. Chemicals used for the preparation of Tyrode's solution, Tris-NaCl-EDTA (TNE) buffer (0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid detergent solution (0.15 M NaCl, 0.08 N HCl, 0.1% Triton-X 100, pH 1.2), acridine orange (AO) staining buffer (0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 0.1 M citric acid, pH 6.0) as well as propidium iodide (PI) were purchased from Sigma-Aldrich Co. (Buchs, Switzerland). The fluorochromes CELLTRACE Calcein Violet AM, Fluo-4 AM and 1,1',3,3,3',3'-Hexamethylindodicarbocyanine iodide [MITOPROBE $DiIC_1(5)$] were obtained from ThermoFisher Scientific Inc. (Waltham, U.S.A.), while AO and phycoerythrin-conjugated agglutinin of *Arachis hypogea* (PE-PNA) were purchased from Polysciences Europe GmbH (Eppelheim, Germany) and GeneTex Inc. (Irvine, U.S.A.), respectively.

The purchased fluorescent probes were diluted and used for sperm staining in form of working solutions as described here: 25 μg calcein violet AM/52 μL dimethyl sulfoxide (DMSO; 1.21 μM); 10 mg PI/5 mL double-distilled water (2.99 mM PI); 1 mg/mL PE-PNA; 50 μg Fluo-4 AM/225 μL DMSO (2 μM); 10 μM $DiIC_1(5)$ in DMSO (0.015 μM).

Sperm Chromatin Structure Assay™ (SCSA™)[1]. The SCSA was performed to evaluate the susceptibility of sperm to acid-induced DNA fragmentation at 0h, using a COULTER EPICS XL flow cytometer driven by EXPO32 ADC XL 4 COLOR software (Beckman Coulter Inc., Krefeld, Germany). In short, 400 μL of acid detergent solution were added to 200 μL of semen previously diluted with TNE buffer to a final concentration of 1 to 2×10^6 sperm/mL. Following the mixing of the sample for 30 s, 1.2 mL of AO staining solution (6.0 μg AO/mL AO staining buffer) were added and stained samples were assessed by flow cytometry after exactly 3 min. Cells were excited by a 488-nm argon laser and the emitted green and red fluorescence was captured by means of a 525/20 and a 620/15 band-pass BP filter, respectively. In total, 10000 cells were analyzed for each sample at a flow rate of 200 cells/sec. Flow cytometric data analysis was performed using the 4.07.0005 version of FCS EXPRESS 4 Flow Cytometry Research Edition software (De Novo Software, Glendale,

U.S.A.). The mean value and SD of the DNA fragmentation index (DFI) as well as the percentage of cells with high DFI (%DFI) were computed as previously described by Evenson and Jost (2001).

Multicolor flow cytometric assay[2]. The multicolor assay was performed using the CytoFLEX Flow Cytometer V5-B5-R3 operated by the CytExpert Software for CytoFLEX version 2.1 (Beckman Coulter Inc., Nyon, Switzerland), as previously described by Bucher et al. (2019). The flow cytometer included five channels from the violet (405 nm) laser, five channels from the blue (488 nm) laser, and three channels from the red (638 nm) laser. The violet, blue, and red solid-state diode lasers operated with a power of 80 mW, 50 mW, and 50 mW, respectively. Flow rate was set to 60 µL/min and 500 to 1000 events/sec; for each sample 10000 cells were analyzed. A fluorescent panel consisting of calcein violet AM, PI, PE-PNA, Fluo-4 AM and $DiIC_1(5)$ was employed for the simultaneous evaluation of intracellular esterase activity, plasma membrane integrity, acrosomal status, intracellular Ca^{2+} levels, and mitochondrial membrane potential of sperm, respectively. The laser and band-pass (BP) filters used for the excitation and detection of emission signal of each fluorophore, respectively.

For the examination of each sperm sample with the multicolor assay, sperm was diluted to a concentration of 1.2×10^6 sperm/mL with Tyrode's solution at a final volume of 244.75 µL in a 250-µL reaction well of a 96-well plate. Just prior to the performance of the assay, the fluorescent probes were combined in a master mix solution consisting of 0.375 µL calcein violet AM, 1.5 µL PI, 0.5 µL PE-PNA, 2.5 µL Fluo-4 AM, and 0.375 µL $DiIC_1(5)$ per reaction well. Thus, 5.25 µL of master mix were added to each reaction well. After 15 min of incubation at 38 °C, sperm were analyzed by flow cytometry.

The % size of the sperm sub-population ($C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$) simultaneously exhibiting the following features was quantified: high esterase activity, intact plasma membrane, PE-PNA-unstained acrosome, low $[Ca^{2+}]_i$ and high mitochondrial membrane potential. Values of PMAI were also determined.

2.3. Analysis of sperm small non-coding RNA profile

2.3.1. Sperm RNA extraction

Sperm homogenization. Total RNA was extracted from cryopreserved bovine sperm using a modified heated TRIzol® Reagent-based protocol (TRIzol® Reagent, Invitrogen, ThermoFisher Scientific, Waltham MA, U.S.A.). Sperm samples of each bull (four cryopreserved ejaculates per bull, one straw per ejaculate) were thawed on ice and pooled immediately after thawing; the sperm pellet was separated from semen extender after centrifugation at 5000 × g for 5 min (4 °C). Homogenization of harvested sperm was performed according to the procedure previously described by Rauber (2008). In short, the sperm pellet was re-suspended with 1 mL of ice-cold hypotonic somatic cell lysis buffer (10 mM Tris HCl, 50 mM KCl, 2.5 $MgCl_2$, 4 mM DTT, 0.05% w/v SDS, 0.5 v/v Triton-X 100; pH 7.4), incubated on ice for 10 min and thereafter centrifuged at 5000 × g for 5 min (4 °C). After discarding the supernatant, the harvested pellet was washed with 1 mL of ice-cold 1× PBS (Invitrogen, ThermoFisher Scientific, Vilnius, LT) and centrifuged at 5000 for 5 min (4 °C). Then, re-harvested pellet was suspended with 1.5 mL of ice-cold TRIzol® Reagent (1.5 mL TRIzol®/1.2×10^6 sperm). In order to disrupt the plasma membrane of sperm, the TRIzol®-sperm suspension was passed four times through a 25G needle and vigorously mixed for 30 sec.

Total RNA extraction. Samples were centrifuged at 12000 × g for 10 min (4 °C). The harvested pellet consisted of insoluble material, such as membranes, polysaccharides and high molecular weight DNA (Rauber, 2008) and was, therefore, discarded; RNA was contained in the supernatant, which was transferred in a new tube, and heated at 65 °C for 10 min while being mixed at 600 rpm. Right after, 200 µL chloroform/mL TRIzol® were added and samples were vigorously mixed for 30 sec. Following a 3-minute incubation at room temperature, samples were centrifuged (12000 × g, 4 °C, 15 min) and 500 µL of the aqueous phase containing the spermatozoal total RNA were separated and transferred to a new tube that includes 500 µL of 100% Isopropanol/mL TRIzol ®. Total RNA was allowed to precipitate for 45 min at room temperature after the addition of 10 µg RNase-free glycogen/mL TRIzol®. The RNA pellet was harvested after centrifugation at maximum relative centrifugal force (rcf) for 30 min (4 °C), twice washed with 1 mL 75% Ethanol/mL TRIzol® (centrifugation in between the washing steps at maximum rcf for 5 min, 4 °C) and re-suspended with 10 µL pyrogen free DEPC-treated water (Invitrogen, Carlsbad, CA). Then, the pellet was dissolved after 10 min incubation on ice.

DNase-treatment. Collected spermatozoal RNA was treated with RNase-free recombinant DNase I (Invitrogen, ThermoFisher Scientific, Waltham MA, U.S.A.) according to the protocol provided by the manufacturer. Shortly, 1 µl 10× DNase I reaction buffer and 1 µl DNase I (1 U/µl) were added per 10 µl of DEPC-treated water used for the re-suspension of the RNA pellet, and RNA samples were incubated for 15 minutes at room temperature. DNase was inactivated by addition of 1 µl of 25 mM EDTA and heating at 65 °C for 10 min, and was removed through a second precipitation step. Following the addition of 10 µg Glycogen/mL TRIzol®, the tube was filled with RNase-/DNase-free water to 100 µL. Thereafter, 10 µL 3 M NaOAc (sodium acetate) (Sigma-Aldrich) and 100 µL 100% Isopropanol (Sigma-Aldrich) were added according to 1/10 volume. After the incubation of samples at room temperature for 45 min, twice the washing steps with 75% Ethanol (Sigma-Aldrich) was performed as described above. Finally, the RNA pellet was suspended in 10 µL of RNase-/DNase-free water (Gibco, Life Technologies), incubated for 10 min on ice and right after for 10 min at 55 °C before being used for downstream analysis.

2.3.2. Total RNA quality control

Each sample was subjected to quality control regarding the quantity, purity and integrity of harvested RNA, using spectrometric evaluation (NanoDrop® 3300 Fluorospectrometer, ThermoFisher Scientific Inc., V 2.8 software, U.S.A.), and an electrophoretic assay (Agilent BioAnalyzer 2100 system, Agilent Technologies, Santa Clara, CA). The isolated total RNA samples were stored at -80 °C until used for library preparation.

2.3.3. Small RNA Library Preparation

Using a total RNA input of 1 ng, small RNA libraries were prepared with the NEXTflex® Small RNA Sequencing Kit v3 for Illumina® Platforms (Bioo Scientific, U.S.A) according to the manufacturer's instructions. A sufficient amount of ~150 bp product was detected after 22 cycles of polymerase chain reaction (PCR) amplification, but also a considerable amount of ~130 bp adapter-only product was observed in the analysis of the small RNA libraries with an Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, U.S.A.). To reduce the amount of adapter-only products, a polyacrylamide gel size selection was performed. A hand-cast 10% polyacrylamide gel was prepared with the following reagents:

0.8 mL of 50× Tris-acetate-EDTA (TAE), 29.2 mL of H_2O, 0.25 gr ammonium persulfate (APS) 25% dissolved in 1 mL H_2O, 32 mL of N,N,N,N'-tetramethylenediamine (TEMED) which is acting as a catalyst and 10 mL of 40% acrylamide. As a next step, all the reagents were combined well in a beaker for the gel solution, except for the TEMED and APS and were degassed for 15 min. Just prior to pouring, TEMED and APS were added to the solution to polymerize in a prepared cast tray of two glass plates and two spacers. The solution was poured 1 cm bellow the teeth of the comb and it was left to cast for 1 hour. After 1 hour when it is solid, the comb was removed and positioned to stand in the gel stand and 1× TAE buffer was poured. Gel wells were washed with a pipette. Then, 5 µL of 10 bp (2 µg/lane) ready-to-load DNA ladder (Gibco BRL, Life Technologies) and 18 µL of sample and 2 µL of 6× loading dye (ThermoFisher Scientific, LT) were loaded. Samples were loaded slowly to allow them to settle evenly on the bottom of the well. After loading the samples, the gel was started at 100 V and left to run overnight. Then, the voltage was increased up to 300 V for 1 hour until the dye bands were arriving the bottom of the gel. After 1 hour, the gel was removed carefully from the glass plate and stained with SYBR Gold. Forty milliliters of 1× TAE and 4 µL SYBR Gold were prepared. The gel was stained on a shaker for 1 hour. The area between 147-165 bp was cut out on a UV transilluminator (ChemiDoc™ MP Imaging System, Bio-Rad) using a clean scalpel. The gel slices were placed into a clean 1.5 mL tube and crushed thoroughly with a disposable pestle.

Purification from the gel slice. 300 µL of elution buffer was added to the crushed gel slices. To obtain as much gel as possible, the pestle was also washed in the tube with the buffer. Washed gel slices were incubated around 3.5 hours at room temperature on a shaker at 400 rpm. The eluate including crushed gel was transferred carefully to spin columns (Millipore Centrifugal Filter units). After centrifugation at 16000 rcf for 2 min, spin filters were disposed. Then, 50 µL of NEXTflex Cleanup Beads and 350 µL of isopropanol were added and incubated at room temperature for 10 min then vortexed and spinned down for 10 sec. The supernatant was separated into the clean tubes according to volume to hold magnetic plate and magnetized for 2 min or until the solution appeared clear. After discarding the supernatant, 950 µL of freshly prepared 80% ethanol was added and incubated for 30 seconds, then all of the supernatant was removed. This washing step was repeated twice and thereafter samples were dried for 3 minutes. Then, samples were removed from the magnetic stand and bead pellets were re-suspended in 13 µL of resuspension buffer. Finally, the samples were incubated for 2 min and magnetized for 3 minutes thereafter 12 µL of supernatant, which was the sequencing library, was transferred to clean 1.5 mL tubes.

2.3.4. Sequencing and bioinformatics analysis

Small RNA libraries were sequenced at the Functional Genomics Center Zurich (FGCZ) as one pool of 18 barcode-tagged samples on one lane of an Illumina HiSeq 2500 instrument (75 bp single-end reads). Analysis of the obtained fastq files was performed on a local Galaxy server installation (Blankenberg et al., 2010) as previously described (Bick et al., 2018; Alminana et al., 2018). Briefly, the tool ‘clip adapter sequences’ was used to remove adapter sequences. Non-clipped sequences were discarded. Removal of PCR duplicates was based on the four random nucleotides on each side of the cDNA inserts that were introduced during ligation of the 5’ and 3’ RNA adapters during library preparation. All identical cDNA sequences containing the same four nucleotides at the ends were removed by using the tool ‘collapse’. Afterwards, the four random bases were cut at each side of the sequences and unique sequences and their read counts were obtained by running the tool ‘collapse’ again. Then, a count table was generated by joining the lists of all samples in one table. The

sequences with neglectable read counts, mainly derived from sequencing errors were removed by the use of the count per million (cpm) per sample filtering tool. Then, the cutoff was set to 42.84 cpm corresponding to an average of 20 reads per library for at least 5 out of 18 libraries. After that, the annotation of small RNA sequences was performed using Basic Local Alignment Search Tool (BLAST). The BLAST databases involved bovine and human sequences from miRBase (release 22.1), Rfam 14.1, transcript sequences from Ensembl and National Center for Biotechnology Information (NCBI), including non-coding RNAs, and predicted piRNA sequences.

The analysis of differentially expressed (DE) miRNAs was performed using Bioconductor package EdgeR (Robinson et al., 2010). An adjusted P value (false discovery rate, FDR) of 10% was used as a threshold to determine DE miRNAs. Subsequently, miRNet tool (http://www.mirnet.ca/) was used to find target genes of candidate miRNAs and to look for biological processes and pathways that are overrepresented in their target genes (Fan et al., 2016). In addition, following the identification of target genes of miRNAs, the biological DataBase network bioDBnet (http://biodbnet.abcc.ncifcrf.gov) allowed us to match gene symbols with Entrez Gene IDs (bovine and putative homolog orthologs) (Mudunuri et al., 2009). Gene ontology (GO) enrichment analysis (biological process, molecular function, cellular localization) and pathway analysis were conducted by using online tool 'the Database for Annotation, Visualization and Integrated Discovery (DAVID)' (https://david.ncifcrf.gov) (Huang et al., 2009a, 2009b).

2.4. Statistical analysis

Three out of the 18 bulls (two LF and one HF bulls) were not included in the statistical analysis because of their outlier miRNA expression levels (see *Results, Figure 1*). The statistical analysis was performed using the R Language for Statistical Computing version 3.1 (The R Development Core Team, 2019).

***Descriptive statistics*.** The set of continuous variables that was used as input for statistical analysis included: a) sperm quality traits (progressive motility, %DFI, PMAI sperm, $C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$ sperm), b) fertility measures (NRR_{conv}, NRR_{ss}, Δ_{NRR}), and c) miRNA expression levels (cpm). The mean value, standard deviation, min and max values were reported as descriptive measures of sperm quality and fertility traits. The association between miRNA expression levels and sperm quality traits or fertility variables was tested using the Spearman's rank correlation coefficient (r_s) at 0.05 significance level. In this case, r_s values were computed at bull level, i.e. between the cpm of individual miRNAs and the mean value of sperm quality traits across ejaculates of the same bull.

Robust regression. Forward model selection was performed to identify the subset of miRNA whose expression made the most valuable contribution to explaining the variance of NRR_{ss}. A series of linear models including a maximum of five predictors were built using the *regsubsets* function in the *leaps* package for R (Lumley, 2020). The linear model with the best overall fit was selected based upon the values of the Bayesian Information Criterion. After determining the five most important miRNA, the relationship between NRR_{ss} and the latter was assessed with robust regression. The robust regression approach for RNA-seq data has been suggested by Seo et al. (2016) as a way to overcome the issue of influential observations in experiments of small sample size. Shortly, the *rlm* function of the *MASS* package for R was applied for fitting the regression line with an MM-estimator (Venables and Ripley, 2002), with the expression levels of the selected miRNA and NRR_{ss} functioning as predicting and response variables of the model M_{ss}, respectively; because of the small sample size in our

study, model parameters were determined using the Wald test. Similar to model M_{ss}, a second robust regression line (M_{conv}) was fit with the expression of the five selected miRNAs functioning as predictors of NRR_{conv}. Values of the outcome and predicting variables in M_{ss} and M_{conv} were mean-centered and scaled by one SD, in order to compare the standardized b coefficients between the two models.

3. RESULTS

3.1. Descriptive statistics

Sperm quality traits. Descriptive statistics (mean value±SD, min and max values) of sperm quality characteristics are presented in Table 2. The samples examined in our study were commercially produced doses that had already passed the post-cryopreservation quality control before being released in the market; thus, not surprisingly PMAI values of both fertility groups were higher than the commonly applied threshold of 40% (45.96%±8.63% and 48.98%±8.75% for the LF and HF bulls, respectively). As demonstrated in Table 2, LF bulls showed lower $C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$ (31.12%±6.66% and 35.08%±8.19% for the LF and HF group, respectively) but similar %DFI values (4.01%±1.59% and 4.57%±2.21% for the LF and HF group, respectively).

Small RNA sequencing data. In total, 48170 to 1070345 reads were identified in each sample (279763±235489 reads per sample). More than 50% of the total reads (50.78% to 72.62%) were 18- to 30-nucleotide long (Figure 1). Across the 18 samples, 4209 unique sequences were identified after filtering of sequences with neglectable read counts. Alignment of unique sequences against bovine and human non-coding and coding sequences revealed 683 sncRNA transcripts in total, with the number of uniquely mapped reads per sample ranging between 5788 and 277775 reads. Eighty-five miRNAs were identified across the 18 analyzed samples. A subset of 55 out of the 85 miRNAs was found in common with miRNAs detected in our previous studies on 30 bovine sperm samples from two cohorts of bulls (Malama et al., 2018). The 10 most abundant miRNAs are presented in Figure 2. MiR-34b-3p and miR-100-5p were the two most highly expressed miRNAs, with their relative abundancy reaching approximately 30% in total (Figure 2). Counts per million reads of the 85 detected miRNAs in the pooled sperm sample of each bull are available in Supplemental Table S1.

Correlation between sperm quality traits, miRNA expression levels and fertility data. The r_s coefficients (and their respective P values) describing the relation between the 85 miRNA expression levels and sperm quality or fertility traits are presented in Supplemental Table S2. The values of NRR_{conv} were moderately related ($0.5<|r_s|\leq 0.7$, $P<0.05$) to four out of the 85 identified miRNAs (miR-2340, miR-26a, miR-425-5p, miR-151-5p), while NRR_{ss} was significantly correlated with the cpm of nine miRNAs (Supplemental Table S2). In particular, the expression levels of miR-9-5p, miR-34c, miR-449a, miR-2483-5p and miR-21-5p were negatively related to NRR_{ss} ($-0.657\leq r_s\leq -0.515$, $P<0.05$; Supplemental Table S2). A moderate positive correlation was detected between NRR_{ss} and the cpm of miR-423-5p, miR-1246, miR-92a and miR-5193-5p ($0.521\leq r_s\leq 0.693$, $P<0.05$; Supplemental Table S2). Interestingly, the expression levels of the nine above mentioned miRNAs were not related to the NRR_{conv} or other sperm quality traits, with exception of miR-423-5p and miR-1246 that were correlated to %DFI ($r_s=-0.576$, $P=0.031$) and $C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$ sperm at 0h ($r_s=0.541$, $P=0.046$), respectively (Supplemental Table S2).

Table 2. Descriptive statistics (mean value±SD, min and max values) of sperm quality traits determined in 60 cryopreserved unsorted ejaculates produced from 15 bulls (four ejaculates per bull). Bulls were assigned to two groups (low fertility, LF; high fertility, HF) based on their fertility performance after artificial insemination with sex-sorted sperm.

	LF				HF				Total			
	n	Mean±SD	Min	Max	n	Mean±SD	Min	Max	n	Mean±SD	Min	Max
Progressive motility (%)	28	36.21±13.33	9.50	66.60	32	37.82±7.95	19.50	58.60	60	37.07±10.83	9.50	66.60
PMAI sperm (%)	28	45.96±8.63	20.66	58.98	32	48.98±8.75	26.52	63.67	60	47.69±8.83	20.66	63.67
$C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$ sperm (%)	28	31.12±6.66	17.19	45.54	32	35.08±8.19	19.27	55.19	60	33.39±7.82	17.19	55.19
Mean DFI	28	201.91±3.50	198.53	214.29	32	201.02±5.99	179.30	214.94	60	201.40±5.10	179.30	214.94
SD of DFI	28	33.52±11.60	20.53	73.77	32	36.65±9.91	20.95	65.63	60	33.14±10.76	20.53	73.77
%DFI (%)	28	4.01±1.59	2.05	8.65	32	4.57±2.21	2.04	10.14	60	4.33±1.99	2.04	10.14

n, number of ejaculates; PMAI, percentage of sperm with intact plasma membrane and unstained acrosome; $C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$, percentage of sperm with high esterase activity, intact plasma membrane, unstained acrosome, low intracellular Ca^{2+} levels and high mitochondrial membrane potential; DFI, DNA fragmentation index; %DFI, percentage of sperm with high DNA fragmentation-index

Figure 1. Number of total reads (light blue) and reads with length of 18-30 nucleotides (dark blue) in unsorted sperm samples of 18 bulls (four cryopreserved ejaculates pooled per bull). Bulls A to I and bulls J to R showed low and high fertility after artificial insemination with X-bearing cryopreserved sperm, respectively.

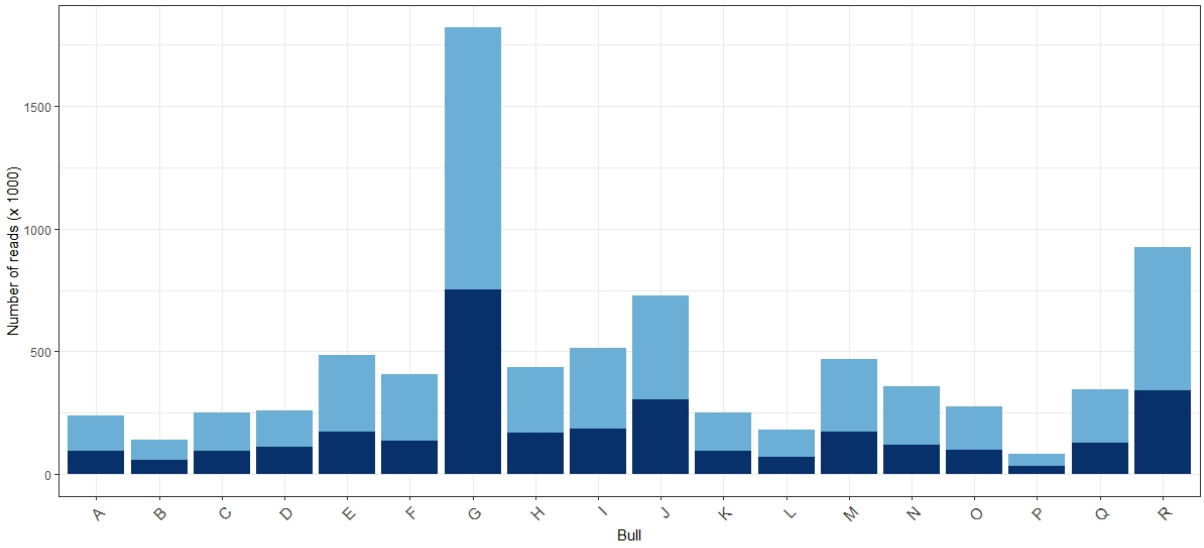
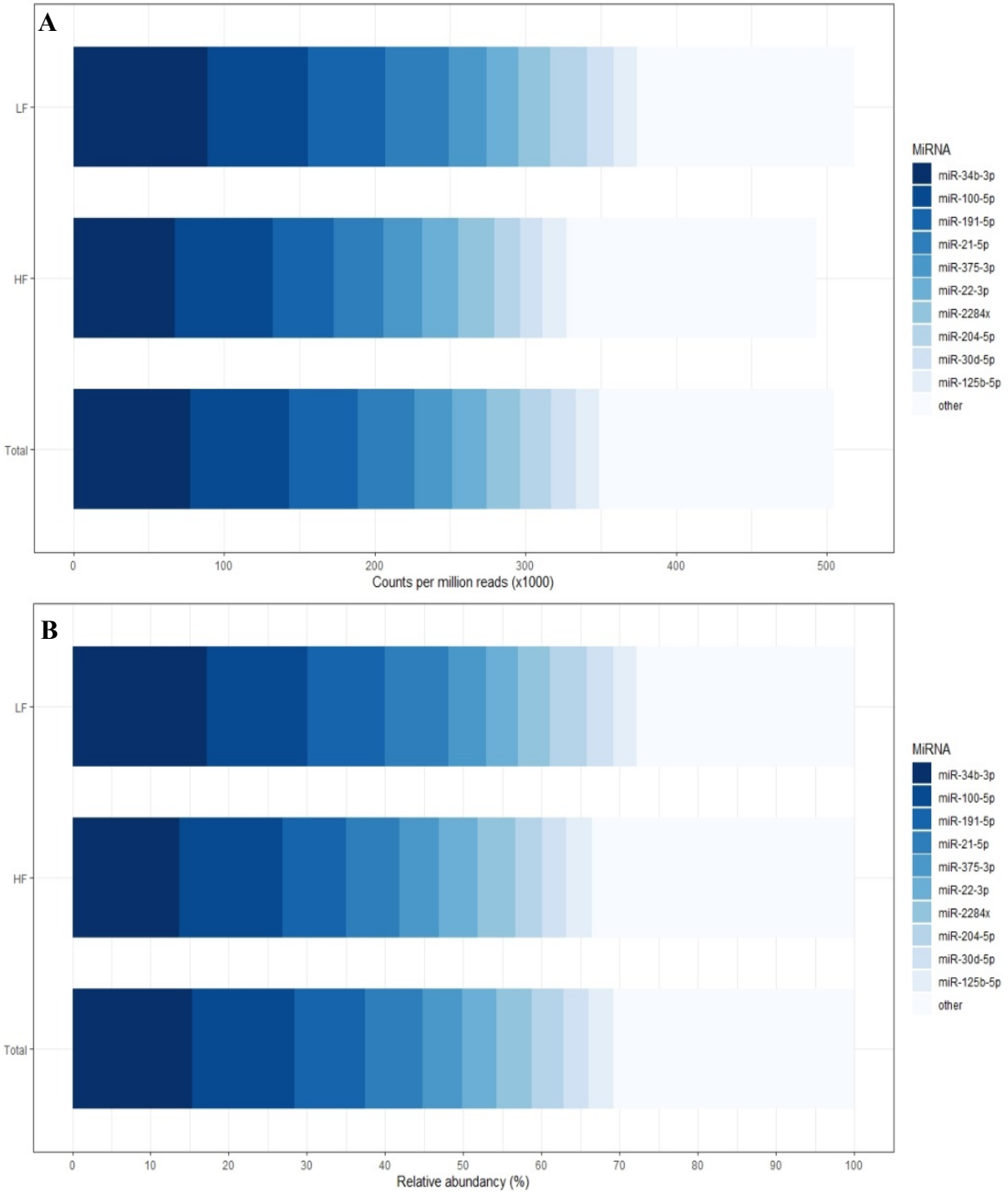


Figure 2: Cumulative bars demonstrating the counts per million reads (panel A) and relative abundancy (panel B) of the top 10 miRNAs detected in unsorted sperm samples of seven and eight bulls (four cryopreserved ejaculates pooled per bull), with low (LF) and high (HF) fertility after artificial insemination with sex-sorted sperm, respectively.



3.2. Differentially expressed miRNAs of the group comparison

Two out of 85 miRNAs were differentially expressed between samples of the LF and HF group; miR-10a-5p and miR-9-5p (FDR<10% in both cases). In particular, miR-9-5p was downregulated and miR-10a-5p was upregulated in HF vs. LF sperm samples (-1.26 and 1.31 log fold change, respectively). Functional annotation for target genes of the two DE miRNAs was performed using the DAVID functional annotation clustering tool. The highest enrichment scores were related to binding (2.35), metabolic process, gene expression, cell proliferation (1.90), cell cycle regulation (1.67), regulation of response to stress, cellular component organization (1.63), cell cycle process (1.54), reproductive process and cell adhesion (1.49), organelle organization (1.47), and cellular localization (1.37). Relevant data are presented in Supplemental Table S3.

3.3. Robust regression

Forward model selection revealed five miRNAs with the highest contribution to the prediction of NRR_{ss}: miR-34c, miR-7859, miR-342, miR-106b-5p and miR-92a. Thus, the following robust regression line (M_{ss}) was fit:

$$NRR_{ssi} = a + b_1[miR\text{-}34c]_i + b_2[miR\text{-}7859]_i + b_3[miR\text{-}342]_i + b_4[miR\text{-}106b\text{-}5p]_i + b_5[miR\text{-}92a]_i + e_i$$

where NRR_{ssi} is the estimated value of NRR_{ss} for individual i, a the intercept of the regression line, $b_{1\text{-}5}$ the coefficients of the respective linear regressors, [miR-x] the expression levels (cpm) of the selected miRNA, and e the additive error term of the model. The variance inflation factor (VIF) of each regressor was computed to evaluate the multicollinearity of expression levels in the subset of the five miRNAs. The regression coefficients b (±SEM) and their respective P and VIF values are shown in Table 3. Values of NRR_{ss} were negatively related to the expression levels of miR-34c (b=-0.011±0.003, P=0.002) and miR-342 (b=-0.005±0.001, P=0.022), while the miR-7859 appeared to have a positive effect on NRR_{ss} (b=0.041±0.014, P=0.009; Table 3). The effect of miR-106b-5p expression levels on the latter was proven not significant (b=-0.016±0.010, P=0.122; Table 3). Although NRR_{ss} values were positively related to cpm of miR-92a, this trend was not statistically significant (0.016±0.005, P=0.058; Table 3). The NRR_{ss} values predicted with robust regression for each of the five miRNAs are demonstrated in Figure 3A; the observed NRR_{ss} values in relation to the performance group are presented in Figure 3B.

In a following step, we tried to explore whether the five selected miRNAs made an actual contribution to the variance of the outcome variable NRR_{ss} or indirectly affected the sire's performance after sex-sorting through its overall fertility status. Therefore, NRR_{conv} was modeled as a function of the five miRNAs (model M_{conv}). The standardized b coefficients and the confidence intervals of models M_{ss} and M_{conv} are graphically demonstrated in Figure 4. The regression coefficients describing the relation of the five selected miRNAs to NRR_{conv} were closer to zero, while their 95% confidence intervals crossed the vertical zero-threshold line, apparently indicating non-significance of the M_{conv} model parameters. Therefore, it was confirmed that the five selected miRNAs had a direct effect on the fertilizing potential of sex-sorted sperm and did not affect its performance through the general fertility status of the bull.

Table 3. Parameters (estimate of coefficients b±SEM, respective t statistic and P values) of the robust regression line describing the relation between non-return rate values after artificial insemination with sex-sorted sperm and the expression levels of five miRNAs selected through forward model selection. The variance inflation factor (VIF) of each regressor was computed to evaluate the multicollinearity of expression levels in the subset of the five miRNAs.

	Estimate of coefficient b (±SEM)	t statistic	P value	VIF
(*Intercept*)	69.648±6.131	11.360	<0.001	
miR-34c	-0.011±0.003	-4.461	0.002	2.184
miR-7859	0.041±0.014	3.350	0.009	2.464
miR-342	-0.005±0.001	-2.773	0.022	2.513
miR-106b-5p	-0.016±0.010	-1.707	0.122	1.683
miR-92a	0.016±0.005	2.171	0.058	1.623

SEM, standard error of the mean; VIF, variance inflation factor

Figure 3. Robust regression lines fitted for the prediction of the 56-day non-return rates (NRR_{ss}) of seven low- (LF) and eight high-fertility (HF) bulls after artificial insemination with X-bearing cryopreserved sperm (left-side panel a); predicted NRR_{ss} values are presented for each one of the five miRNAs that were included as independent variables in the robust regression model. Grey-shaded areas represent the 95% confidence intervals of the robust regression lines. The expression levels (counts per million, cpm) of five sperm miRNAs are presented conditional on the two fertility groups (right-side panel b).

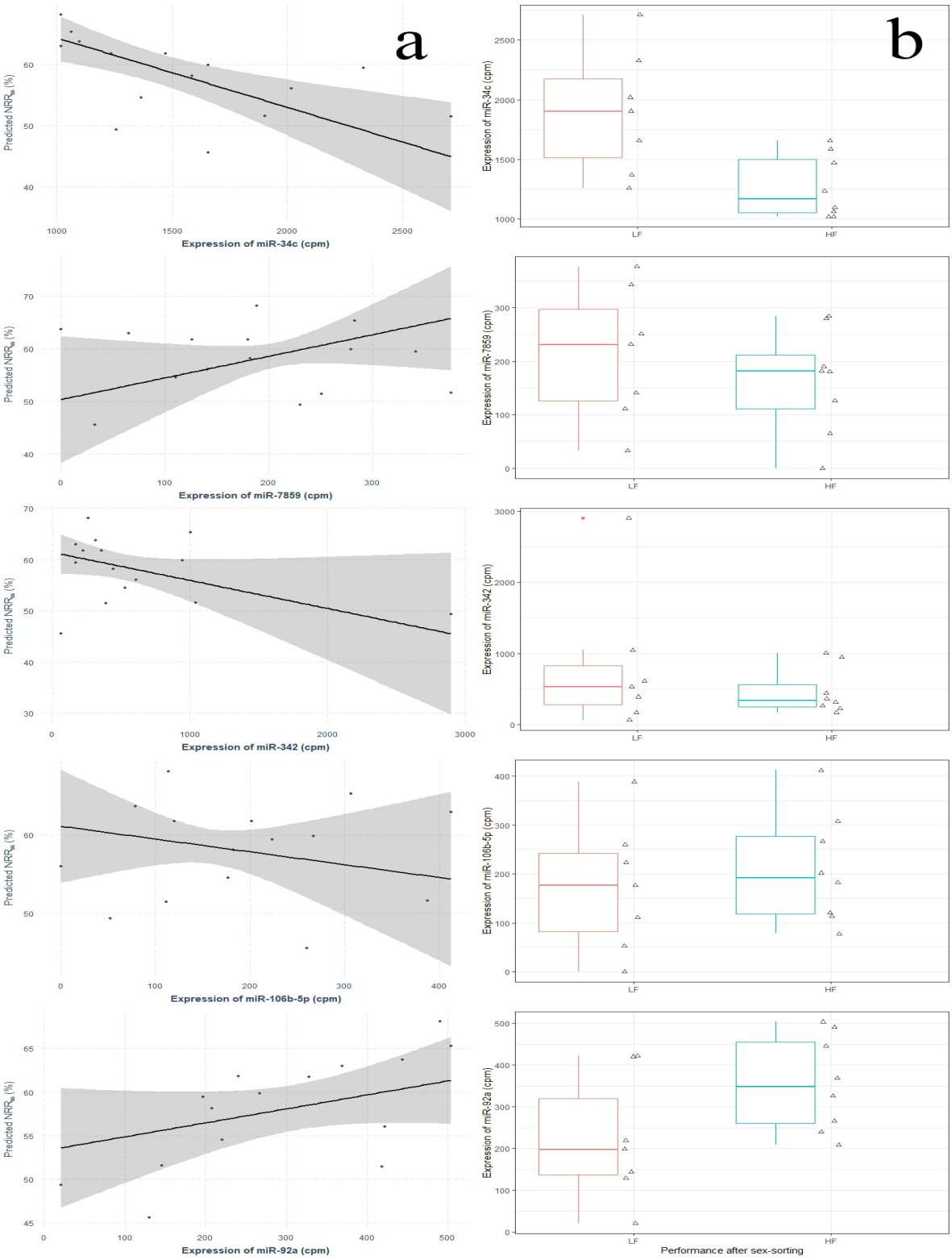
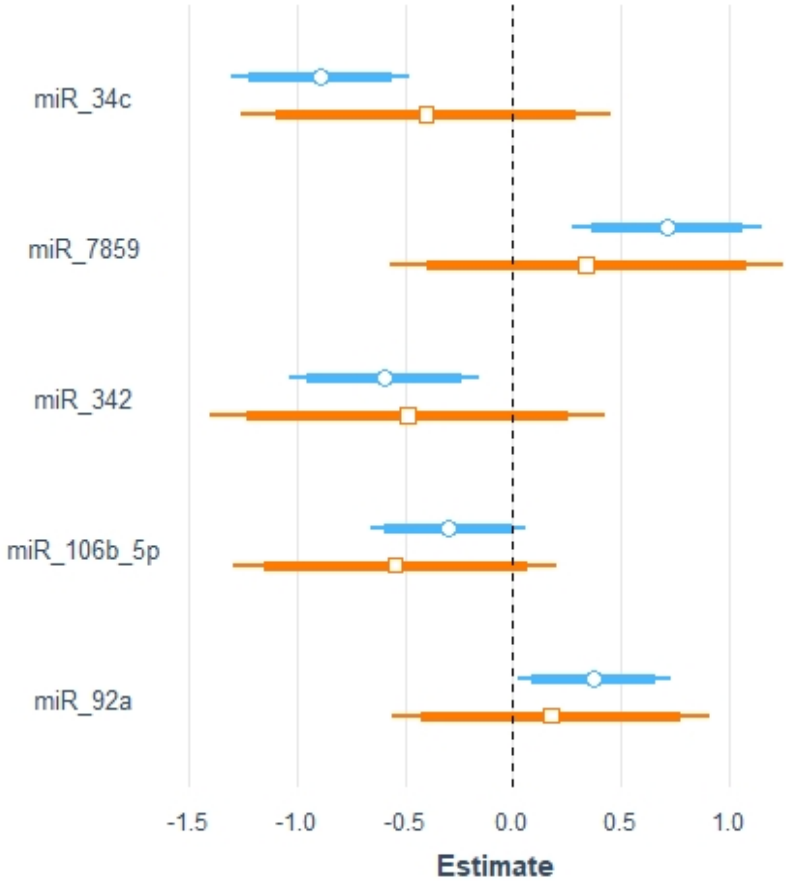


Figure 4. Standardized b coefficients for the robust regression models describing the relation between the expression levels of five sperm miRNAs and the 56-day non-return of 15 bulls recorded after artificial insemination with unsorted (orange) and sex-sorted (blue) cryopreserved sperm. Transparent points represent the mean of the standardized coefficient b, the horizontal line the 95% CI and the thicker part of the line the 90% CI of the estimated coefficients.



4. DISCUSSION

The use of sex-sorted sperm in bovine assisted reproduction is constantly expanding; however, it is frequently observed that bulls with high fertility after AI with conventional sperm may not perform optimally when sex-sorted doses are used for inseminating either cows or heifers. In the present study, we explored the relation between the miRNA profile of conventionally produced semen doses and the fertility status of the bull after AI with sex-sorted sperm.

Our analysis revealed a wide variety of miRNAs in bovine sperm, with miR-34b-3p and miR-100-5p comprising approximately 30% of the analyzed sperm miRNAome. The most abundant miRNA, miR-34b-3p, was present in both HF and LF bulls with a relative abundancy of approximately 15%. Similar values have been previously reported for bovine sperm in other studies (Tscherner et al., 2014; Capra et al., 2017). It has been shown that transcripts of the miR-34b/c cluster are preferentially expressed in the testis and play a crucial role in sperm chromatin condensation in the stage of pachytene spermatocytes and round spermatids (Yuan et al., 2015). Based on the outcome of robust regression analysis, miR-34c but not miR-34b-3p was highlighted as a deciding predictor of NRR_{ss}. Even though the two miRNAs are co-transcribed from a common cluster (miR-34b/c) on bovine chromosome 15, their expression and function can largely vary within the same cell type (Tscherner et al., 2014). This could probably explain the relation of miR-34c but not of miR-34b-3p with NRR_{ss} in our study.

Our results suggested a negative correlation between miR-34c expression levels and the fertility of sperm after sex-sorting. MiR-34c has been detected in sperm of several species, including the equine (Das et al., 2013), porcine (Chen et al., 2017), murine (Nixon et al., 2015) and human (Krawetz et al., 2011; Abu-Halima et al., 2014; Pantano et al., 2015), and is considered as one of the most abundant miRNAs in bull sperm and male germ cells (Sellem et al., 2020; Tscherner et al., 2014; Stowe et al., 2014;). MiR-34c profoundly plays a role in the growth, differentiation and apoptosis of the male germ cell line through regulation of the transforming growth factor beta and the notch signaling pathways (Bouhallier et al., 2010). Individuals not able to express the seed sequence of miR-34c (i.e. lacking miR-34c and miR-449 simultaneously) have lower sperm concentration and impaired sperm kinetics (Yuan et al., 2015). In the same direction, Capra et al. (2017) reported elevated miR-34c expression levels in the high-motile fraction of cryopreserved bovine sperm selected by means of Percoll density gradient centrifugation. In our study, miR-34c cpm were related neither to the functional traits of conventional sperm nor to NRR_{conv}. This could be apparently attributed to the low between-bull variability of the latter. However, it is not easy to explain why sperm with lower miR-34c expression performs better after sex-sorting, as indicated by our analysis. Recent studies have highlighted the importance of an intense co-expression and target network of miRNAs involved in the manifestation of complex phenotypic traits including bovine fertility (Sellem et al., 2020; Fang et al., 2018). In the light of this information, one could speculate that pre-sorting elevated miR-34c expression indirectly affects the fertilizing ability of sperm after sorting, possibly through alterations in the expression or function of interrelated miRNAs. Interestingly, the profile of sperm miR-34c is susceptible to semen processing and particularly cryopreservation (Zhang et al., 2017). Future split-ejaculate experiments focusing on the effect of sex-sorting on sperm miRNA profile could elucidate the role of miR-34c in the fertility of sex-sorted sperm.

As suggested by our results, miR-342 was also included in the predictors of NRR_{ss}. This miRNA has been previously identified in various bovine tissues (Long et al., 2009; Li et al., 2015) as well as in the testes and sperm of other mammalian species (Liu et al., 2012; Xiong, 2014; Muñoz et al., 2015). MiR-342 belongs to the long list of miRNAs whose relative

abundance substantially changes during epididymal maturation (Nixon et al., 2015), a process that renders sperm their ability to capacitate once deposited in the female genital tract. Furthermore, miR-342 can block the lipogenesis-cholesterogenesis pathway (Li et al., 2013) which is directly linked to the capacitation of mature sperm (Cross, 1998; Travis et al., 2002). Premature capacitation-like changes of sperm structure and function during sorting are counted among the main causes for the reduced longevity of sex-sorted sperm (Bucci et al., 2012; Carvalho et al., 2010, 2018). In our study, miR-342 expression levels in conventional sperm samples were negatively correlated with NRR_{ss} but not with NRR_{conv} or the inducibility of acrosome reaction of conventional sperm (flow cytometrically assessed after challenging sperm with calcium ionophore A23187 and dual PI/PNA staining; data not shown). Thus, any potential effect of miR-342 expression on the fertilizing ability of X-bearing sperm was apparently not linked to the capacitation status of spermatozoa before sorting.

Remarkably, our analysis pointed out miR-7859, a miRNA that had not been previously described in bovine germ cells or sperm, as one of the five predictors of NRR_{ss}. It has been previously documented as part of the microRNAome of the bovine (Le Guillou et al., 2014) and caprine mammary gland (Mobuchon et al., 2015), but information about its functional role are rather scarce. Based on our results, the expression level of miR-7859 appeared to have a positive predictive value for NRR_{ss}; however, miR-7859 transcripts were on average more abundant in LF than in HF bulls. At this point, one should note that miR-7859 and miR-106b-5p, another predictor of NRR_{ss} values in our robust regression model, were listed among the five least abundant miRNAs in the examined samples. It is most likely that the low expression levels of miR-7859 in sperm samples of both groups along with the relatively small sample size of our experiment are responsible for these inconsistent observations.

Regarding miR-92a, its expression levels were distinctly lower in conventional sperm produced by bulls with sub-optimal performance after sex-sorting. However, the positive predictive value of miR-92a for NRR_{ss} was proven marginally not significant. Even when the weakly expressed miR-7859 and miR-106b-5p were excluded from robust regression analysis, the effect of miR-92a on NRR_{ss} was still not significant (data not shown). MiR-92a and miR-106b-5p are members of the multifunctional paralog gene clusters miR-17-92 and miR-106b-25, respectively, and are considered essential for the undisrupted progress of spermatogenesis (Tong et al., 2012; Xie et al., 2016; Hurtado et al., 2018). MiR-17-92- and miR-106b-25-deficient mice display extended loss of spermatogonia and spermatogonial stem cells, and, consequently, testicular atrophy and reduced sperm production (Tong et al., 2012; Xie et al., 2016). The small number of sperm that reach the epididymis of miR-17-92-mutant animals exhibit normal morphology and motility characteristics (Xie et al., 2016), which implies that miR-92a might have an effect on male fertility but in a manner not directly linked to conventional sperm quality characteristics. Interestingly, Tong et al. (2012) showed that the expression of miR-106b-25 cluster increases dramatically in the germ cells of miR-17-92 knockout male mice, while sperm quality of the latter remains unchanged. This observation urged the authors to suggest that the members of the two paralog clusters miR-17-92 and miR-106b-25 function in a redundant way in sperm cells. Apparently, impairment of the function of either of the two miRNA clusters can be compensated by the other, so that no phenotypic alterations are observed in the produced sperm (Tong et al., 2012). In the light of this information, one could attribute the inconsistent effect of miR-92a and miR-106b-5p on NRR_{ss} to the well documented functional interrelation of the two miRNAs and potential collinearity issues in the robust regression model.

When we compared the miRNA profiles between sperm samples of the two artificially created groups LF and HF, two out of the 85 sperm miRNAs were found to be differentially expressed. MiR-9-5p and miR-10a-5p were down- and upregulated in the HF and LF sperm, respectively. Transcripts of miR-10a have previously been detected in mature bovine sperm

(Capra et al., 2017; Stowe et al., 2014), while miR-9-5p has been frequently reported in porcine sperm (Dai et al., 2019; Zhang et al., 2017; Kasimanickam and Kastelic, 2016). It has been shown that both miRNAs are upregulated in porcine sperm after cryopreservation (Dai et al., 2019; Zhang et al., 2017); however, there is no information regarding the effect of sex-sorting on miR-10a-5p and miR-9-5p profiles. In the bull, miR-10a-5p is upregulated in sperm with low motility (Capra et al., 2017). Furthermore, overexpression of miR-10a-5p in late spermatogenesis can adversely affect the DNA repair capacity of murine spermatocytes leading to severe testicular atrophy and infertility in adulthood (Gao et al., 2019). Based on the literature described above, the upregulation of miR-10a-5p is linked to impaired sperm quality, which makes it difficult to explain the overexpression of miR-10a-5p in sperm samples obtained from HF bulls in our study.

The production of cryopreserved sex-sorted sperm comes with inherent stress, like the elevated sorting pressure and the subsequent freezing steps, which substantially impairs sperm quality. Changes of sperm morphometry (Carvalho et al., 2013) and function (Suh et al., 2005) are well documented consequences of mechanical stress during sorting. Although there is some indication for the importance of miRNA for sperm "freezability" (Dai et al., 2019), their role in the resistance of sperm against mechanical stimuli has not been studied yet. Several miRNAs mediate metabolic changes induced by mechanical stress in other types of cells (Luna et al., 2011; Hu et al., 2014; Yuan et al., 2017), including miR-92a, miR-34c and miR-21-5p that were correlated with NRR_{ss} values in our study. Nevertheless, the contribution of miRNA, if any, to the response of bovine sperm to sorting stress is still to be clarified.

In the present study, we intentionally selected bulls with uniform NRR_{conv} but with diverse fertility after AI with sex-sorted sperm, in order to avoid the masked effect of a sire's general fertility status on his performance after sex-sorting. The examined semen doses had successfully passed the post-thaw quality control before being used for commercial AI in the field, and laboratory assessed traits of conventional sperm showed relatively low between-bull variability. Given the limited variance of NRR_{conv} and sperm quality characteristics, it is not surprising that only few miRNAs were related to the above-mentioned parameters. In contrast to differential expression analysis, robust regression enabled the analysis of sperm miRNAome in relation to continuous fertility and sperm variables without losing within-group phenotypic variation; however, limitations in the interpretation of the results due to the small sample size of our study must be considered.

In conclusion, we were able to detect 85 miRNAs in conventional cryopreserved semen doses collected from proven sires that were in parallel used for the production of sex-sorted sperm. Our analysis revealed that NRR_{ss} values were related to the expression levels of five out of the 85 miRNAs (miR-34c, miR-342, miR-7859, miR-106b-5p and miR-92a) but not to the post-thaw quality characteristics of conventional sperm. This finding raises questions regarding the mechanisms by which these miRNAs could affect the performance of sperm after sex-sorting. Whether they are responsible for subtle alterations of spermatozoal phenotypes that are only manifested after sex-sorting or they specifically interfere with the performance of X-bearing sperm, remains to be clarified. Studies with higher numbers of samples and split-ejaculate experiments focusing on miRNA profiles of sex-sorted compared to conventional sperm are apparently necessary to validate the suitability of sperm miRNAs as markers for the fertilizing potential of sex-sorted sperm.

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SUPPLEMENTAL TABLES

1. SUPPLEMENTAL TABLE S1

Expression levels (counts per million) of 85 miRNAs detected in samples of unsorted cryopreserved sperm produced from 15 proven sires (four ejaculates pooled per bull). Bulls were classified in two groups based on their fertility after artificial insemination with sex-sorted sperm (i.e. high and low fertility; HF and LF group, respectively).

Breed	Group
Holstein-Friesian	LF-A
	LF-D
	LF-E
	LF-H
	HF-M
	HF-Q
Red Holstein	LF-B
	HF-J
	HF-K
Simmental	LF-C
Swiss Fleckvieh	LF-I
Brown Swiss	HF-L
	HF-N
	HF-R
Limousin	HF-O

	Bull	A	B	C	D	E	H	I	J	K	L	M	N	O	Q	R
	Group	LF	LF	LF	LF	LF	LF	LF	HF	HF	HF	HF	HF	HF	HF	HF
miRNA																
let-7a-5p		4224	2019	4162	11062	5893	5979	3185	3275	7098	10408	7370	5527	4796	6838	5182
let-7b		4256	2677	1425	651	3024	958	1803	805	6034	11110	8389	3665	3927	3053	1607
let-7f		520	282	391	556	265	158	92	179	174	1171	340	270	113	126	323
let-7g		390	47	196	955	883	388	224	688	217	885	235	691	982	126	291
miR-100-5p		36909	102041	39354	76933	84575	81329	45244	97218	48773	89013	63716	51219	51995	46149	74869
miR-106b-3p		0	282	335	84	44	388	92	246	87	130	157	330	0	227	260
miR-106b-5p		260	0	112	53	177	388	224	267	412	182	78	120	113	202	307
miR-10a-5p		1852	1268	2039	168	1810	1152	750	1882	5318	7988	4312	1923	2077	2347	2355
miR-10b		2664	329	1816	346	1523	1310	1224	2165	3386	5776	1960	1893	1737	1463	2496
miR-11975		9195	8312	3435	3201	27037	7022	3882	2983	5709	7442	23782	57017	25223	7696	17538
miR-11987		3152	2066	1927	11	640	619	1487	914	2518	859	680	2283	944	1236	1481
miR-122		0	0	84	63	265	364	197	234	239	0	0	180	76	25	284
miR-1234		227	235	335	147	155	243	145	50	174	52	1045	330	1171	707	126
miR-1246		3412	3381	5670	53	2119	1916	4382	4138	6946	7129	8076	2223	4154	3961	6875
miR-125a		227	1221	447	1868	1744	970	671	1306	1758	911	680	481	717	732	914
miR-125b-5p		13224	20568	10502	13308	17899	17610	15621	21059	14391	19385	14452	18204	10195	14029	15223
miR-1260b		357	704	810	304	1148	1407	513	396	391	286	653	691	1397	1438	827
miR-128		1300	1174	1006	1417	2163	1686	1277	2153	1498	1743	784	1202	869	1236	1488
miR-1306		552	939	307	1322	750	958	711	592	478	520	523	1051	755	908	213
miR-132-3p		163	376	251	84	441	740	382	196	261	130	105	120	227	404	284
miR-132-5p		0	47	419	63	221	315	171	104	239	338	340	300	415	151	354
miR-146a-5p		1820	1221	2961	6801	1037	1892	1263	9278	1519	1431	706	511	1510	3659	3363
miR-148a		292	986	559	262	971	691	487	847	369	468	209	511	151	833	709

miR-151-5p	1949	2301	3966	4030	4017	6852	3066	5023	2301	4319	3450	3124	1964	2574	3252
miR-15a-5p	1137	282	531	483	331	667	500	367	825	572	392	631	1095	732	370
miR-15b-5p	2892	3569	5670	3338	3818	4160	5040	3108	5014	3539	3241	3725	3436	6586	3867
miR-16a-5p	7473	4602	6201	20634	4039	6779	5883	9553	5991	4007	4417	2794	7627	10042	6946
miR-16b-5p	9162	4320	9273	17423	6290	9799	8107	12031	8053	6219	3554	4025	8609	12994	9986
miR-186-5p	2567	2630	2290	1553	3598	3638	2435	4401	3994	4293	1542	4386	2266	2448	4898
miR-191-5p	46169	19112	33321	116375	34695	66715	43112	60123	35728	41111	40404	23041	30661	40093	50204
miR-1937	1365	1127	2598	220	3951	2365	1579	672	803	755	1829	1532	1435	2372	2213
miR-200a	812	282	559	210	684	400	329	471	738	1353	497	300	227	328	512
miR-200b	292	1033	1536	1690	3090	2207	1079	2140	2257	4371	1882	1682	869	1665	1937
miR-204-3p	422	423	531	262	486	473	369	113	847	104	836	1592	227	858	410
miR-204-5p	12184	32542	13295	58828	14876	33048	8804	37992	10744	10824	8677	14660	9855	24525	18168
miR-211	227	892	223	1406	132	303	105	484	130	156	131	150	189	530	158
miR-21-5p	44512	42216	46700	36850	40081	33424	51417	37829	32993	45560	34811	20608	27942	37444	28634
miR-22-3p	26902	22164	38628	20739	11609	18762	8541	18005	17712	8769	9800	15140	37533	49933	35454
miR-2284aa	195	282	307	189	662	170	487	242	521	781	732	781	151	404	788
miR-2284f	1852	1456	2207	3012	1236	1601	1895	3917	2692	2056	1150	1472	2492	3684	3764
miR-2284m	747	2442	1620	315	1700	1079	684	563	825	1093	2195	1322	1246	1539	1087
miR-2284x	18130	27659	25110	3894	40036	18640	12120	10734	18862	23886	30211	28268	21221	22633	36178
miR-2285aj-5p	227	1738	335	84	441	267	171	200	174	312	784	210	566	530	347
miR-2285am	455	0	28	0	441	85	158	134	391	286	732	451	0	227	480
miR-2285bf	552	0	475	430	221	85	434	651	586	416	314	391	0	328	331
miR-2285bh	357	751	307	84	243	376	132	179	261	442	862	240	529	429	158
miR-2340	7570	4461	3743	535	5231	2498	8436	793	1780	2290	10689	3304	6835	5198	3835
miR-23a	130	704	615	1008	773	837	645	809	651	546	183	210	566	807	591
miR-2483-5p	260	329	335	703	66	376	276	184	109	234	0	150	151	379	268
miR-25-3p	715	1221	922	1364	1170	1698	882	1222	217	598	1098	1232	1057	782	1449
miR-26a	715	94	642	2078	1368	1419	684	1485	1237	2446	941	1112	906	555	1504

miR-27a-3p	3022	986	2989	4776	2031	2183	3422	4435	3126	1795	784	1232	1926	3179	2922
miR-27b	3152	2113	2262	5111	3222	2886	3422	5832	3885	3903	1934	1983	2643	2574	3126
miR-2887-3p	390	141	56	0	309	170	250	92	347	26	418	751	302	303	402
miR-2888-3p	6206	3240	5530	661	3929	3469	2751	380	2431	1405	7370	10454	7967	5046	3434
miR-2889-3p	8772	986	6620	577	8277	3129	6251	284	9703	3122	16177	7600	8836	10295	3347
miR-29a	260	657	1229	1574	905	776	671	1727	912	286	680	541	378	782	906
miR-30a-5p	7635	11693	8240	17507	11521	13037	12160	12528	9485	10070	7657	7210	8383	10421	11065
miR-30d-5p	11891	15966	15334	24791	17811	18519	18214	20112	18884	14337	10794	10815	11894	18318	16065
miR-30e-5p	3249	3992	4190	2687	3289	2959	4435	4672	4168	3799	2300	3575	2568	4718	2693
miR-32	1787	141	1145	1595	905	1116	2198	2007	1433	1275	1281	541	831	1539	1937
miR-335	3412	1784	4106	7756	4635	5300	5198	6783	3299	3591	3267	2674	2077	4945	5938
miR-340-5p	260	0	782	903	1964	813	698	1035	434	1717	993	481	378	278	772
miR-342	65	611	391	2897	530	1043	171	947	174	442	314	361	264	227	1008
miR-3432a	1040	1691	531	2697	2781	1965	1224	2645	2301	911	2744	1833	415	3734	1457
miR-34b-3p	44609	114673	64212	143181	70450	126165	62010	101536	47232	67885	57156	72818	45236	78672	67427
miR-34c	1657	2019	2709	1260	1368	1904	2329	1656	1020	1587	1098	1472	1020	1236	1063
miR-3620-5p	715	704	1313	168	2053	594	882	255	1302	625	2561	3184	1964	1463	551
miR-375-3p	23198	23808	33405	13434	36969	28015	15845	14893	23942	19515	28696	24933	26847	35022	29240
miR-423-5p	163	47	391	105	530	534	171	255	239	312	653	931	982	934	362
miR-425-5p	4061	5964	4832	4366	5981	8587	3448	8681	6642	6115	2404	6309	3436	7342	6623
miR-449a	487	235	447	220	309	230	329	225	152	234	105	421	113	202	126
miR-5193-5p	390	141	782	241	662	558	763	33	586	286	1176	1953	2303	807	465
miR-6089-3p	4906	4837	5111	535	4458	1856	3711	839	4081	1587	4730	14930	3436	2801	3386
miR-6526	2924	1268	6983	1658	2494	4572	2198	768	1975	1613	2091	4566	2756	4365	4442
miR-6529a	0	0	196	441	66	279	118	63	326	26	183	150	151	379	142
miR-658-3p	390	282	978	105	1104	582	434	163	478	572	1464	1833	1020	656	1370
miR-660	98	329	223	273	353	218	132	234	347	234	209	240	0	76	213
miR-7	3866	5259	2542	3600	3929	6246	2040	6846	2127	4996	3659	2433	4305	3659	3599

miR-7859	33	141	251	231	110	376	342	280	65	182	0	180	189	126	284
miR-7865	292	282	615	105	839	497	276	50	369	234	2404	871	793	404	496
miR-92a	130	423	419	21	221	146	197	267	369	208	444	240	491	328	504
miR-93	552	423	1536	987	1810	1698	1277	809	1498	1015	1281	1863	831	984	1032
miR-9-5p	520	704	475	378	419	158	632	259	195	286	0	60	151	252	244
miR-99a-5p	2534	3334	3156	1826	3443	4827	1790	4472	2475	3643	2979	2463	3889	2044	2654

2. SUPPLEMENTAL TABLE S2

Spearman's rank correlation coefficients r_s (and respective P values) between bull fertility parameters, the post-thaw quality traits and the miRNA expression levels (counts per million) of unsorted cryopreserved bovine sperm. Fertility data of 15 bulls were recorded in form of an annual 56-day non-return rate after >500 first services with unsorted (NRR_{conv}) or sex-sorted (NRR_{ss}) sperm, respectively. The percentage of sperm with intact plasma membrane and unstained acrosome (PMAI), the percentage of sperm with high esterase activity, intact plasma membrane, unstained acrosome, low intracellular Ca^{2+} levels and high mitochondrial membrane potential ($C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$), the mean and standard deviation of the sperm DNA fragmentation index (DFI) and the percentage of sperm with high DFI were flow cytometrically assessed at 0 hours post-thaw. Significant r_s values are highlighted.

	Spearman's rank correlation coefficients (highlighted when respective adjusted P value<0.05)								
	Fertility traits			**Sperm quality traits at 0 hours**					
miRNA	**NRR_{conv}**	**NRR_{ss}**	**Relative difference of NRR_{conv} and NRR_{ss}**	**PMAI**	**$C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$**	**Progressive motility**	**Mean DFI**	**SD of DFI**	**%DFI**
miR-10a-5p	-0.215	0.325	0.465	0.371	0.618	0.159	0.072	0.077	0.345
miR-9-5p	0.192	-0.531	-0.676	-0.135	-0.060	0.167	0.020	-0.063	0.208
let-7b	-0.029	0.271	0.325	0.042	0.379	-0.010	-0.136	-0.044	0.243
miR-10b	-0.380	0.182	0.364	0.336	0.694	-0.006	0.151	0.043	0.406
miR-11975	0.237	0.375	0.333	-0.435	-0.419	-0.238	-0.505	-0.518	-0.436
miR-423-5p	0.155	0.524	0.529	-0.132	-0.265	-0.183	-0.460	-0.394	-0.576
miR-2284aa	0.112	0.447	0.482	-0.018	-0.039	0.008	-0.285	-0.304	-0.097
miR-2285am	0.028	0.241	0.266	-0.448	-0.202	-0.409	-0.274	-0.288	0.116
miR-34c	-0.146	-0.515	-0.525	0.122	0.089	0.286	0.226	-0.189	-0.094
miR-449a	-0.253	-0.657	-0.654	-0.402	-0.279	-0.334	-0.021	-0.464	0.014
miR-1246	0.118	0.521	0.555	0.248	0.541	0.206	-0.172	-0.033	0.226
miR-92a	0.461	0.693	0.609	0.098	0.215	0.382	-0.492	-0.259	-0.213
miR-2483-5p	-0.286	-0.525	-0.491	0.130	-0.266	0.053	0.218	0.185	-0.147
miR-658-3p	0.205	0.467	0.450	-0.334	-0.362	-0.191	-0.627	-0.694	-0.611

let-7a-5p	-0.353	-0.059	0.071	0.182	-0.053	-0.257	0.114	0.213	0.013
miR-132-3p	-0.067	-0.139	-0.131	0.338	0.025	0.349	0.252	-0.115	-0.326
miR-132-5p	0.025	0.491	0.547	0.320	0.248	0.108	-0.347	-0.431	-0.581
miR-186-5p	-0.358	0.256	0.448	0.406	0.413	0.277	0.333	0.044	0.158
miR-204-5p	-0.341	-0.388	-0.306	0.135	-0.253	0.167	0.552	0.566	0.024
miR-21-5p	-0.035	-0.515	-0.568	0.041	0.191	0.061	0.224	0.122	0.316
miR-2284f	-0.147	0.267	0.366	0.356	0.206	0.051	0.220	0.495	0.270
miR-2887-3p	0.317	0.404	0.332	-0.476	-0.368	-0.348	-0.550	-0.525	-0.174
miR-34b-3p	-0.364	-0.402	-0.310	0.245	-0.235	0.361	0.615	0.461	-0.162
miR-1234	0.442	0.482	0.361	-0.280	-0.181	-0.170	-0.560	-0.200	-0.380
miR-3620-5p	0.385	0.425	0.331	-0.382	-0.360	-0.261	-0.594	-0.549	-0.357
miR-5193-5p	0.459	0.525	0.406	-0.249	-0.204	-0.265	-0.695	-0.482	-0.505
miR-30a-5p	-0.150	-0.318	-0.297	0.305	-0.110	0.168	0.508	0.587	0.043
let-7f	-0.494	-0.318	-0.164	0.032	0.209	-0.029	0.151	0.017	0.133
let-7g	-0.169	-0.018	0.043	-0.062	-0.006	-0.340	0.097	0.228	-0.077
miR-106b-5p	-0.346	0.046	0.188	0.471	0.503	-0.063	0.437	0.180	0.382
miR-1306	0.032	-0.243	-0.292	-0.105	-0.479	-0.023	0.119	0.181	-0.207
miR-148a	-0.129	-0.080	-0.022	0.226	-0.076	0.527	0.388	0.030	-0.088
miR-15a-5p	-0.023	-0.033	-0.051	-0.159	0.166	-0.461	-0.282	-0.208	0.139
miR-191-5p	-0.395	-0.414	-0.322	0.070	-0.204	-0.283	0.459	0.551	0.074
miR-200b	-0.434	0.105	0.308	0.521	0.372	0.234	0.423	0.214	0.075
miR-204-3p	0.131	0.233	0.214	-0.237	-0.419	-0.145	-0.361	-0.489	-0.189
miR-211	-0.102	-0.380	-0.393	-0.013	-0.354	0.150	0.290	0.477	0.050
miR-2284x	0.165	0.293	0.273	-0.243	-0.213	0.110	-0.458	-0.656	-0.448
miR-2285aj-5p	0.381	0.132	0.005	-0.266	-0.158	0.593	-0.446	-0.253	-0.396
miR-2285bf	-0.332	-0.176	-0.064	0.034	0.214	-0.370	0.361	0.341	0.784
miR-2285bh	0.205	0.204	0.148	-0.194	0.058	0.316	-0.247	-0.149	-0.181
miR-2340	0.580	0.186	-0.024	-0.588	-0.273	-0.323	-0.613	-0.396	-0.211
miR-23a	-0.107	-0.126	-0.093	0.576	0.084	0.362	0.469	0.501	-0.009
miR-25-3p	-0.140	-0.115	-0.078	-0.145	-0.448	0.137	0.143	-0.015	-0.649

miR-26a	-0.530	-0.048	0.160	0.383	0.277	-0.129	0.479	0.439	0.127
miR-27a-3p	-0.225	-0.281	-0.232	0.205	0.051	-0.274	0.421	0.569	0.450
miR-2888-3p	0.286	0.251	0.159	-0.558	-0.415	-0.330	-0.695	-0.719	-0.486
miR-2889-3p	0.366	0.321	0.211	-0.428	-0.228	-0.468	-0.596	-0.434	-0.067
miR-29a	-0.222	-0.156	-0.083	0.243	-0.087	0.068	0.502	0.542	0.187
miR-30d-5p	-0.181	-0.285	-0.245	0.441	0.003	0.131	0.552	0.602	0.254
miR-335	-0.361	-0.259	-0.149	0.190	-0.180	-0.189	0.540	0.549	0.087
miR-342	-0.291	-0.333	-0.266	0.080	-0.295	-0.032	0.339	0.422	-0.116
miR-3432a	-0.070	0.061	0.106	-0.022	-0.373	-0.061	0.298	0.385	0.170
miR-425-5p	-0.565	-0.012	0.222	0.553	0.272	0.453	0.664	0.283	0.127
miR-7859	-0.185	-0.056	0.015	0.505	0.171	0.330	0.348	0.172	-0.326
miR-7865	0.278	0.344	0.278	-0.405	-0.305	-0.214	-0.428	-0.325	-0.359
miR-1937	0.078	-0.034	-0.072	-0.235	-0.407	-0.145	-0.293	-0.624	-0.568
miR-100-5p	-0.250	-0.035	0.076	0.330	0.205	0.568	0.639	0.555	0.032
miR-106b-3p	-0.386	-0.010	0.150	0.344	-0.036	0.577	0.311	-0.143	-0.394
miR-11987	0.092	-0.099	-0.154	-0.235	0.104	-0.055	-0.274	-0.444	0.342
miR-122	-0.134	0.109	0.182	0.385	0.121	0.075	0.337	0.067	-0.101
miR-125a	-0.005	-0.034	-0.028	0.355	0.074	0.169	0.402	0.545	0.313
miR-125b-5p	-0.252	-0.011	0.111	0.270	0.231	0.527	0.652	0.395	0.253
miR-1260b	0.150	0.241	0.207	0.033	-0.246	0.092	-0.263	-0.361	-0.681
miR-128	-0.472	-0.225	-0.056	0.313	0.237	0.070	0.707	0.437	0.347
miR-146a-5p	-0.364	-0.144	-0.017	0.194	0.056	-0.001	0.582	0.720	0.326
miR-151-5p	-0.665	-0.303	-0.078	0.422	0.124	0.198	0.747	0.299	-0.212
miR-15b-5p	0.123	0.123	0.096	0.384	-0.006	0.160	-0.144	-0.230	-0.112
miR-16a-5p	-0.164	-0.308	-0.295	-0.005	-0.273	-0.271	0.219	0.499	0.103
miR-16b-5p	-0.299	-0.289	-0.219	0.171	-0.131	-0.242	0.330	0.478	0.121
miR-200a	-0.463	-0.166	-0.001	0.178	0.521	-0.049	0.238	-0.018	0.409
miR-22-3p	0.022	0.100	0.093	-0.041	-0.171	-0.017	-0.337	-0.263	-0.289
miR-2284m	0.299	0.195	0.109	-0.250	-0.270	0.395	-0.438	-0.548	-0.499
miR-27b	-0.336	-0.173	-0.057	0.342	0.350	-0.092	0.692	0.852	0.636

miR-30e-5p	-0.044	-0.021	0.011	0.418	0.343	0.359	0.350	0.233	0.482
miR-32	-0.127	-0.033	0.013	0.088	0.191	-0.376	0.287	0.495	0.502
miR-340-5p	-0.310	-0.097	0.023	0.132	0.089	-0.141	0.323	0.147	-0.044
miR-375-3p	0.061	0.123	0.110	-0.186	-0.323	-0.055	-0.420	-0.671	-0.522
miR-6526	-0.206	-0.101	-0.045	-0.049	-0.308	-0.110	-0.342	-0.753	-0.641
miR-6529a	-0.070	0.039	0.064	0.286	-0.263	-0.153	0.022	0.169	-0.134
miR-660	-0.195	-0.212	-0.150	0.240	0.024	0.328	0.402	0.134	0.237
miR-7	-0.477	-0.139	0.033	0.226	0.305	0.395	0.696	0.533	0.047
miR-93	-0.107	0.049	0.100	0.170	-0.197	-0.166	-0.027	-0.413	-0.372
miR-99a-5p	-0.408	0.002	0.163	0.330	0.462	0.392	0.513	0.216	-0.113
miR-6089-3p	0.210	0.155	0.094	-0.399	-0.369	-0.137	-0.478	-0.645	-0.257

	P values								
	Fertility traits			**Sperm quality traits at 0 hours**					
miRNA	**NRR_{conv}**	**NRR_{ss}**	**Relative difference of NRR_{conv} and NRR_{ss}**	**PMAI**	**$C_{pos}PI_{neg}PNA_{neg}F_{neg}M_{pos}$**	**Progressive motility**	**Mean DFI**	**SD of DFI**	**%DFI**
miR-10a-5p	0.441	0.237	0.081	0.192	0.019	0.572	0.807	0.793	0.227
miR-9-5p	0.492	0.042	0.006	0.646	0.839	0.553	0.946	0.830	0.477
let-7b	0.918	0.329	0.238	0.886	0.182	0.973	0.644	0.881	0.402
miR-10b	0.163	0.515	0.182	0.240	0.006	0.982	0.607	0.883	0.150
miR-11975	0.396	0.168	0.225	0.120	0.136	0.393	0.065	0.058	0.119
miR-423-5p	0.581	0.045	0.042	0.653	0.360	0.514	0.098	0.163	0.031
miR-2284aa	0.692	0.095	0.069	0.951	0.895	0.976	0.324	0.290	0.742

miR-2285am	0.922	0.386	0.338	0.108	0.489	0.130	0.343	0.317	0.694
miR-34c	0.604	0.050	0.044	0.679	0.763	0.301	0.437	0.517	0.749
miR-449a	0.363	0.008	0.008	0.154	0.335	0.224	0.943	0.094	0.962
miR-1246	0.675	0.046	0.032	0.392	0.046	0.462	0.555	0.910	0.437
miR-92a	0.084	0.004	0.016	0.738	0.460	0.160	0.074	0.370	0.465
miR-2483-5p	0.302	0.044	0.063	0.659	0.357	0.850	0.454	0.527	0.615
miR-658-3p	0.463	0.079	0.093	0.243	0.204	0.496	0.016	0.006	0.020
let-7a-5p	0.197	0.834	0.802	0.533	0.857	0.356	0.699	0.465	0.965
miR-132-3p	0.813	0.622	0.640	0.237	0.933	0.202	0.386	0.695	0.255
miR-132-5p	0.930	0.063	0.035	0.265	0.394	0.700	0.224	0.124	0.029
miR-186-5p	0.190	0.357	0.094	0.150	0.142	0.317	0.245	0.881	0.589
miR-204-5p	0.213	0.153	0.267	0.646	0.382	0.551	0.041	0.035	0.936
miR-21-5p	0.901	0.049	0.027	0.889	0.512	0.828	0.441	0.679	0.271
miR-2284f	0.600	0.336	0.179	0.211	0.480	0.856	0.450	0.072	0.351
miR-2887-3p	0.250	0.135	0.227	0.085	0.196	0.203	0.041	0.054	0.552
miR-34b-3p	0.183	0.137	0.261	0.398	0.418	0.186	0.019	0.097	0.580
miR-1234	0.099	0.069	0.186	0.333	0.536	0.544	0.037	0.494	0.181
miR-3620-5p	0.157	0.114	0.229	0.177	0.206	0.347	0.025	0.042	0.210
miR-5193-5p	0.085	0.044	0.133	0.391	0.485	0.340	0.006	0.081	0.066
miR-30a-5p	0.593	0.248	0.283	0.290	0.708	0.548	0.063	0.027	0.884
let-7f	0.062	0.248	0.558	0.914	0.474	0.918	0.607	0.955	0.651
let-7g	0.547	0.948	0.879	0.832	0.984	0.215	0.743	0.433	0.794
miR-106b-5p	0.207	0.870	0.502	0.089	0.067	0.823	0.118	0.539	0.178
miR-1306	0.911	0.382	0.291	0.721	0.083	0.934	0.685	0.536	0.478
miR-148a	0.647	0.778	0.939	0.438	0.796	0.043	0.171	0.919	0.765
miR-15a-5p	0.934	0.907	0.856	0.587	0.571	0.084	0.329	0.476	0.635
miR-191-5p	0.145	0.125	0.242	0.813	0.483	0.307	0.099	0.041	0.803
miR-200b	0.106	0.709	0.263	0.056	0.191	0.401	0.132	0.462	0.799
miR-204-3p	0.642	0.403	0.443	0.415	0.136	0.605	0.205	0.076	0.517
miR-211	0.718	0.162	0.147	0.966	0.214	0.595	0.314	0.085	0.865

miR-2284x	0.556	0.289	0.325	0.402	0.464	0.698	0.099	0.011	0.109
miR-2285aj-5p	0.161	0.640	0.985	0.359	0.589	0.020	0.110	0.382	0.161
miR-2285bf	0.227	0.531	0.821	0.909	0.463	0.175	0.205	0.233	0.001
miR-2285bh	0.463	0.467	0.598	0.507	0.844	0.251	0.395	0.611	0.536
miR-2340	0.023	0.506	0.932	0.027	0.345	0.240	0.020	0.161	0.470
miR-23a	0.704	0.654	0.740	0.031	0.775	0.185	0.091	0.068	0.975
miR-25-3p	0.619	0.682	0.781	0.622	0.108	0.626	0.625	0.961	0.012
miR-26a	0.042	0.865	0.569	0.176	0.338	0.648	0.083	0.116	0.665
miR-27a-3p	0.420	0.311	0.405	0.481	0.861	0.323	0.134	0.034	0.106
miR-2888-3p	0.301	0.367	0.571	0.038	0.140	0.230	0.006	0.004	0.078
miR-2889-3p	0.179	0.244	0.450	0.127	0.434	0.079	0.024	0.121	0.819
miR-29a	0.426	0.580	0.768	0.403	0.767	0.811	0.067	0.045	0.522
miR-30d-5p	0.519	0.304	0.378	0.115	0.991	0.642	0.041	0.023	0.380
miR-335	0.187	0.351	0.597	0.514	0.539	0.500	0.046	0.042	0.767
miR-342	0.293	0.226	0.337	0.786	0.306	0.911	0.235	0.133	0.694
miR-3432a	0.806	0.829	0.707	0.941	0.189	0.828	0.300	0.174	0.561
miR-425-5p	0.028	0.967	0.427	0.040	0.347	0.090	0.010	0.327	0.666
miR-7859	0.510	0.843	0.957	0.066	0.558	0.230	0.222	0.556	0.256
miR-7865	0.317	0.209	0.316	0.151	0.289	0.444	0.126	0.257	0.208
miR-1937	0.783	0.904	0.798	0.418	0.149	0.606	0.309	0.017	0.034
miR-100-5p	0.368	0.902	0.789	0.250	0.482	0.027	0.014	0.039	0.914
miR-106b-3p	0.155	0.972	0.595	0.228	0.903	0.024	0.279	0.627	0.163
miR-11987	0.745	0.726	0.584	0.418	0.724	0.846	0.343	0.112	0.231
miR-122	0.634	0.698	0.516	0.174	0.680	0.789	0.238	0.820	0.732
miR-125a	0.987	0.903	0.921	0.212	0.802	0.546	0.154	0.044	0.277
miR-125b-5p	0.364	0.969	0.694	0.350	0.428	0.044	0.012	0.162	0.382
miR-1260b	0.595	0.388	0.460	0.912	0.396	0.744	0.364	0.204	0.007
miR-128	0.076	0.420	0.842	0.276	0.415	0.805	0.005	0.118	0.224
miR-146a-5p	0.182	0.608	0.953	0.507	0.850	0.997	0.029	0.004	0.255
miR-151-5p	0.007	0.272	0.782	0.132	0.672	0.480	0.002	0.298	0.467

miR-15b-5p	0.663	0.662	0.734	0.175	0.984	0.568	0.624	0.429	0.703
miR-16a-5p	0.560	0.265	0.286	0.987	0.346	0.328	0.451	0.069	0.725
miR-16b-5p	0.278	0.295	0.432	0.559	0.656	0.385	0.249	0.084	0.681
miR-200a	0.082	0.554	0.997	0.543	0.056	0.863	0.412	0.950	0.146
miR-22-3p	0.939	0.722	0.742	0.890	0.558	0.953	0.239	0.363	0.316
miR-2284m	0.280	0.486	0.699	0.389	0.351	0.145	0.117	0.043	0.069
miR-27b	0.221	0.538	0.840	0.232	0.220	0.745	0.006	0.000	0.015
miR-30e-5p	0.877	0.941	0.968	0.137	0.230	0.189	0.219	0.422	0.081
miR-32	0.653	0.906	0.962	0.764	0.514	0.167	0.320	0.072	0.068
miR-340-5p	0.261	0.731	0.935	0.652	0.763	0.616	0.260	0.616	0.881
miR-375-3p	0.830	0.662	0.697	0.524	0.260	0.846	0.135	0.009	0.056
miR-6526	0.462	0.721	0.873	0.867	0.285	0.696	0.231	0.002	0.014
miR-6529a	0.804	0.891	0.821	0.322	0.364	0.587	0.941	0.564	0.649
miR-660	0.487	0.449	0.594	0.408	0.935	0.232	0.154	0.647	0.416
miR-7	0.072	0.621	0.907	0.436	0.288	0.145	0.006	0.049	0.874
miR-93	0.704	0.864	0.724	0.560	0.500	0.554	0.928	0.143	0.191
miR-99a-5p	0.131	0.995	0.561	0.249	0.096	0.149	0.061	0.457	0.700
miR-6089-3p	0.453	0.582	0.740	0.157	0.194	0.627	0.084	0.013	0.375

3. SUPPLEMENTAL TABLE S3

Functional annotation with the highest enrichment scores for predicted target genes of the two differentially expressed miRNAs (miR-10a-5p and miR-9-5p) in DAVID (Database for Annotation, Visualization and Integrated Discovery) functional annotation clustering tool.

Predicted Target Genes	Gene ID
BCL6	604
CCNG1	900
ETS1	2113
ID4	3400
POU2F2	5452
RAP2A	5911
SRSF1	6426
UGDH	7358
NCOR2	9612
BCL2L11	10018
UHRF1	29128
TRPM7	54822
CNOT6	57472
E2F7	144455
MTX3	345778
SFT2D2	375035

Annotation Cluster 1	Enrichment Score: 2.3472383809101056										
Category	**Term**	**Count**	**%**	**PValue Genes**	**List Total**	**Pop Hits**	**Pop Total**	**Fold Enrichment**	**Bonferroni**	**Benjamini**	**FDR**
GOTERM_MF_FAT	GO:1901363~heterocyclic compound binding	11	68.75	0.002029 9612, 57472, 7358, 144455, 29128, 54822, 2113, 5911, 604, 5452, 6426	13	5971	15478	2.193396287	0.2320462	0.232046	2.33741
GOTERM_MF_FAT	GO:0097159~organic cyclic compound binding	11	68.75	0.002288 9612, 57472, 7358, 144455, 29128, 54822, 2113, 5911, 604, 5452, 6426	13	6052	15478	2.16403986	0.2575484	0.138344	2.63242
GOTERM_MF_FAT	GO:0003676~nucleic acid binding	8	50	0.019568 9612, 57472, 144455, 29128, 2113, 604, 5452, 6426	13	4097	15478	2.324853082	0.9233905	0.16765	20.5574
Annotation Cluster 2	**Enrichment Score: 1.9014833311112287**										
Category	**Term**	**Count**	**%**	**PValue Genes**	**List Total**	**Pop Hits**	**Pop Total**	**Fold Enrichment**	**Bonferroni**	**Benjamini**	**FDR**
GOTERM_BP_FAT	GO:0031325~positive regulation of cellular metabolic process	10	62.5	2.24E-04 9612, 144455, 3400, 29128, 2113, 5911, 10018, 604, 5452, 6426	16	2839	16650	3.665463191	0.2215675	0.117712	0.35808
GOTERM_BP_FAT	GO:0010604~positive regulation of macromolecule metabolic process	10	62.5	2.35E-04 9612, 144455, 3400, 29128, 2113, 5911, 10018, 604, 5452, 6426	16	2856	16650	3.643644958	0.2309683	0.083819	0.37542
GOTERM_BP_FAT	GO:0009893~positive regulation of metabolic process	10	62.5	3.96E-04 9612, 144455, 3400, 29128, 2113, 5911, 10018, 604, 5452, 6426	16	3052	16650	3.40964941	0.3580811	0.104903	0.63286
GOTERM_BP_FAT	GO:0006366~transcription from RNA polymerase II promoter	8	50	5.36E-04 9612, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	1820	16650	4.574175824	0.4508821	0.112981	0.85483
UP_KEYWORDS	Transcription regulation	8	50	6.69E-04 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	2332	20581	4.412735849	0.048327	0.048327	0.69817
UP_KEYWORDS	Repressor	5	31.25	7.18E-04 9612, 144455, 3400, 29128, 604	16	592	20581	10.86412584	0.0517863	0.026237	0.7493
UP_KEYWORDS	Transcription	8	50	7.94E-04 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	2398	20581	4.291284404	0.0570817	0.019401	0.82789
GOTERM_BP_FAT	GO:0010628~positive regulation of gene expression	7	43.75	0.002431 9612, 144455, 3400, 29128, 2113, 5452, 6426	16	1692	16650	4.30518617	0.9343791	0.322349	3.82594
GOTERM_BP_FAT	GO:0051173~positive regulation of nitrogen compound metabolic process	7	43.75	0.003039 9612, 144455, 3400, 29128, 2113, 5452, 6426	16	1767	16650	4.122453311	0.9668254	0.346718	4.76089
GOTERM_MF_FAT	GO:0044212~transcription regulatory region DNA binding	5	31.25	0.003175 9612, 144455, 29128, 604, 5452	13	853	15478	6.978988186	0.3386186	0.128734	3.63575
GOTERM_MF_FAT	GO:0000975~regulatory region DNA binding	5	31.25	0.003216 9612, 144455, 29128, 604, 5452	13	856	15478	6.954529116	0.3421342	0.099395	3.68174
GOTERM_MF_FAT	GO:0001067~regulatory region nucleic acid binding	5	31.25	0.00323 9612, 144455, 29128, 604, 5452	13	857	15478	6.946414146	0.3433089	0.080668	3.69716
GOTERM_BP_FAT	GO:0006357~regulation of transcription from RNA polymerase II promoter	7	43.75	0.003686 9612, 144455, 3400, 29128, 2113, 604, 5452	16	1835	16650	3.969686649	0.9839448	0.31313	5.74568
GOTERM_BP_FAT	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	5	31.25	0.003708 9612, 144455, 3400, 29128, 604	16	748	16650	6.956049465	0.9843483	0.292793	5.78004
UP_KEYWORDS	Nucleus	10	62.5	0.0048 9612, 57472, 144455, 3400, 29128, 2113, 900, 604, 5452, 6426	16	5244	20581	2.452922387	0.2995668	0.085167	4.91141
GOTERM_BP_FAT	GO:2000113~negative regulation of cellular macromolecule biosynthetic process	6	37.5	0.00482 9612, 57472, 144455, 3400, 29128, 604	16	1324	16650	4.715823263	0.9955123	0.340239	7.45076
GOTERM_BP_FAT	GO:0034654~nucleobase-containing compound biosynthetic process	10	62.5	0.005816 9612, 57472, 7358, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4357	16650	2.38839798	0.998537	0.304154	8.92457
GOTERM_BP_FAT	GO:0018130~heterocycle biosynthetic process	10	62.5	0.006354 9612, 57472, 7358, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4411	16650	2.359158921	0.9992019	0.313011	9.71163
GOTERM_BP_FAT	GO:0051254~positive regulation of RNA metabolic process	6	37.5	0.006409 144455, 3400, 29128, 2113, 5452, 6426	16	1415	16650	4.41254417	0.9992496	0.302144	9.79119
GOTERM_BP_FAT	GO:0010558~negative regulation of macromolecule biosynthetic process	6	37.5	0.006467 9612, 57472, 144455, 3400, 29128, 604	16	1418	16650	4.403208745	0.9992971	0.292295	9.87569
GOTERM_BP_FAT	GO:0019438~aromatic compound biosynthetic process	10	62.5	0.00649 9612, 57472, 7358, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4424	16650	2.352226492	0.9993148	0.281921	9.9087
GOTERM_BP_FAT	GO:0006351~transcription, DNA-templated	9	56.25	0.007047 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	3605	16650	2.59795423	0.999634	0.291102	10.7142
GOTERM_MF_FAT	GO:0043565~sequence-specific DNA binding	5	31.25	0.007415 144455, 29128, 2113, 604, 5452	13	1080	15478	5.512108262	0.6199743	0.148923	8.30211
GOTERM_BP_FAT	GO:0031327~negative regulation of cellular biosynthetic process	6	37.5	0.007755 9612, 57472, 144455, 3400, 29128, 604	16	1480	16650	4.21875	0.9998353	0.294224	11.7294
GOTERM_BP_FAT	GO:0010629~negative regulation of gene expression	6	37.5	0.007866 9612, 57472, 144455, 3400, 29128, 604	16	1485	16650	4.204545455	0.9998547	0.288146	11.8879
GOTERM_BP_FAT	GO:0009890~negative regulation of biosynthetic process	6	37.5	0.008299 9612, 57472, 144455, 3400, 29128, 604	16	1504	16650	4.151429521	0.9999109	0.29206	12.5025
GOTERM_BP_FAT	GO:0051172~negative regulation of nitrogen compound metabolic process	6	37.5	0.008346 9612, 57472, 144455, 3400, 29128, 604	16	1506	16650	4.145916335	0.9999155	0.284617	12.5684
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	9	56.25	0.009232 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	3762	16650	2.489533493	0.9999689	0.300841	13.8119
GOTERM_BP_FAT	GO:0097659~nucleic acid-templated transcription	9	56.25	0.009372 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	3771	16650	2.483591885	0.9999734	0.296165	14.0066
GOTERM_MF_FAT	GO:0001012~RNA polymerase II regulatory region DNA binding	4	25	0.009993 9612, 144455, 604, 5452	13	604	15478	7.884870097	0.7290012	0.170158	11.0379
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	6	37.5	0.010669 9612, 144455, 3400, 29128, 2113, 5452	16	1597	16650	3.909674389	0.9999939	0.312768	15.7936
UP_KEYWORDS	Activator	4	25	0.011245 57472, 144455, 604, 5452	16	661	20581	7.784039334	0.5669154	0.112677	11.1625
GOTERM_BP_FAT	GO:0032774~RNA biosynthetic process	9	56.25	0.011658 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	3905	16650	2.398367478	0.999998	0.328086	17.1323
GOTERM_MF_FAT	GO:0003700~transcription factor activity, sequence-specific DNA binding	5	31.25	0.011735 144455, 29128, 2113, 604, 5452	13	1231	15478	4.835968256	0.7844471	0.174543	12.8436
GOTERM_MF_FAT	GO:0001071~nucleic acid binding transcription factor activity	5	31.25	0.011768 144455, 29128, 2113, 604, 5452	13	1232	15478	4.832042957	0.7853848	0.15717	12.8776
GOTERM_BP_FAT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	5	31.25	0.011898 144455, 3400, 29128, 2113, 5452	16	1041	16650	4.998198847	0.9999985	0.3256	17.4546
GOTERM_BP_FAT	GO:0045935~positive regulation of nucleobase-containing compound metabolic process	6	37.5	0.012461 144455, 3400, 29128, 2113, 5452, 6426	16	1658	16650	3.765832328	0.9999992	0.322788	18.2054
UP_KEYWORDS	DNA-binding	6	37.5	0.012483 9612, 144455, 29128, 2113, 604, 5452	16	2050	20581	3.764817073	0.6052665	0.109697	12.32
GOTERM_MF_FAT	GO:0003714~transcription corepressor activity	3	18.75	0.012722 9612, 144455, 3400	13	226	15478	15.804629	0.8107031	0.15333	13.8518
GOTERM_MF_FAT	GO:0000981~RNA polymerase II transcription factor activity, sequence-specific DNA binding	4	25	0.01283 144455, 2113, 604, 5452	13	662	15478	7.194050662	0.8133769	0.141532	13.9615
GOTERM_MF_FAT	GO:0000976~transcription regulatory region sequence-specific DNA binding	4	25	0.014464 144455, 29128, 604, 5452	13	692	15478	6.882169853	0.8495369	0.146011	15.6056
GOTERM_BP_FAT	GO:0031328~positive regulation of cellular biosynthetic process	6	37.5	0.014802 9612, 144455, 3400, 29128, 2113, 5452	16	1729	16650	3.61119144	0.9999999	0.35541	21.2579
GOTERM_BP_FAT	GO:0045892~negative regulation of transcription, DNA-templated	5	31.25	0.015746 9612, 144455, 3400, 29128, 604	16	1130	16650	4.604535398	1	0.358533	22.4582
GOTERM_BP_FAT	GO:0019219~regulation of nucleobase-containing compound metabolic process	9	56.25	0.015832 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4104	16650	2.282072368	1	0.353095	22.5668
GOTERM_BP_FAT	GO:0009891~positive regulation of biosynthetic process	6	37.5	0.015914 9612, 144455, 3400, 29128, 2113, 5452	16	1760	16650	3.547585227	1	0.347803	22.6704
GOTERM_MF_FAT	GO:1990837~sequence-specific double-stranded DNA binding	4	25	0.016395 144455, 29128, 604, 5452	13	725	15478	6.568912467	0.8834078	0.152372	17.5118
GOTERM_BP_FAT	GO:1903507~negative regulation of nucleic acid-templated transcription	5	31.25	0.018069 9612, 144455, 3400, 29128, 604	16	1177	16650	4.42066695	1	0.364555	25.34
GOTERM_BP_FAT	GO:1902679~negative regulation of RNA biosynthetic process	5	31.25	0.018961 9612, 144455, 3400, 29128, 604	16	1194	16650	4.357726131	1	0.372288	26.4192
GOTERM_MF_FAT	GO:0003676~nucleic acid binding	8	50	0.019568 9612, 57472, 144455, 29128, 2113, 604, 5452, 6426	13	4097	15478	2.324853082	0.9233905	0.16765	20.5574
GOTERM_BP_FAT	GO:0008283~cell proliferation	6	37.5	0.019937 57472, 144455, 3400, 29128, 2113, 604	16	1861	16650	3.355051048	1	0.374677	27.5843
GOTERM_BP_FAT	GO:0051253~negative regulation of RNA metabolic process	5	31.25	0.021514 9612, 144455, 3400, 29128, 604	16	1240	16650	4.196068548	1	0.385369	29.4285
GOTERM_MF_FAT	GO:0003690~double-stranded DNA binding	4	25	0.021671 144455, 29128, 604, 5452	13	805	15478	5.91610129	0.9420522	0.172942	22.5196
GOTERM_BP_FAT	GO:0010468~regulation of gene expression	9	56.25	0.022337 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4345	16650	2.155494822	1	0.390826	30.3737
GOTERM_BP_FAT	GO:0051171~regulation of nitrogen compound metabolic process	9	56.25	0.024201 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4404	16650	2.126617847	1	0.403841	32.4714
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-templated	8	50	0.027315 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	3603	16650	2.310574521	1	0.436672	35.8428
GOTERM_BP_FAT	GO:1903506~regulation of nucleic acid-templated transcription	8	50	0.028192 9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	3625	16650	2.296551724	1	0.435288	36.7646
GOTERM_BP_FAT	GO:0045893~positive regulation of transcription, DNA-templated	5	31.25	0.028405 144455, 3400, 29128, 2113, 5452	16	1349	16650	3.857023721	1	0.432041	36.9857

GOTERM_BP_FAT	GO:1903508~positive regulation of nucleic acid-templated transcription	5	31.25	0.028405	144455, 3400, 29128, 2113, 5452	16	1349	16650	3.857023721	1	0.432041	36.9857
GOTERM_BP_FAT	GO:2001141~regulation of RNA biosynthetic process	8	50	0.028967	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	3644	16650	2.284577387	1	0.427365	37.5675
GOTERM_BP_FAT	GO:1902680~positive regulation of RNA biosynthetic process	5	31.25	0.029801	144455, 3400, 29128, 2113, 5452	16	1369	16650	3.800675676	1	0.431212	38.4217
GOTERM_BP_FAT	GO:0045934~negative regulation of nucleobase-containing compound metabolic process	5	31.25	0.029943	9612, 144455, 3400, 29128, 604	16	1371	16650	3.795131291	1	0.427465	38.5659
GOTERM_BP_FAT	GO:0016070~RNA metabolic process	9	56.25	0.033185	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	4648	16650	2.014979561	1	0.430864	41.7742
GOTERM_MF_FAT	GO:0003677~DNA binding	6	37.5	0.035326	9612, 144455, 29128, 2113, 604, 5452	13	2562	15478	2.788326428	0.9906792	0.253395	34.2187
GOTERM_BP_FAT	GO:0051052~regulation of DNA metabolic process	3	18.75	0.04273	144455, 29128, 604	16	370	16650	8.4375	1	0.478763	50.3341
GOTERM_BP_FAT	GO:2000112~regulation of cellular macromolecule biosynthetic process	8	50	0.045969	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	3991	16650	2.085943373	1	0.486532	52.9598
GOTERM_BP_FAT	GO:0010605~negative regulation of macromolecule metabolic process	6	37.5	0.047386	9612, 57472, 144455, 3400, 29128, 604	16	2326	16650	2.684329321	1	0.488619	54.067
GOTERM_BP_FAT	GO:0031324~negative regulation of cellular metabolic process	6	37.5	0.048848	9612, 57472, 144455, 3400, 29128, 604	16	2345	16650	2.662579957	1	0.495117	55.1841
GOTERM_BP_FAT	GO:0034645~cellular macromolecule biosynthetic process	9	56.25	0.050887	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	5012	16650	1.868640263	1	0.497198	56.6993
GOTERM_BP_FAT	GO:0010556~regulation of macromolecule biosynthetic process	8	50	0.052951	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452	16	4107	16650	2.027027027	1	0.499327	58.1839
GOTERM_MF_FAT	GO:0003682~chromatin binding	3	18.75	0.056284	9612, 29128, 604	13	504	15478	7.086996337	0.9994637	0.357887	49.0649
GOTERM_BP_FAT	GO:0009892~negative regulation of metabolic process	6	37.5	0.063845	9612, 57472, 144455, 3400, 29128, 604	16	2522	16650	2.475713719	1	0.551771	65.2611
GOTERM_MF_FAT	GO:0003712~transcription cofactor activity	3	18.75	0.06615	9612, 144455, 3400	13	552	15478	6.470735786	0.9998632	0.373918	54.9329
GOTERM_BP_FAT	GO:0010467~gene expression	9	56.25	0.069481	9612, 57472, 144455, 3400, 29128, 2113, 604, 5452, 6426	16	5306	16650	1.765100829	1	0.571829	68.4652
GOTERM_MF_FAT	GO:0000977~RNA polymerase II regulatory region sequence-specific DNA binding	3	18.75	0.07679	7144455, 604, 5452	13	601	15478	5.943171637	0.9999692	0.405118	60.5664
GOTERM_MF_FAT	GO:0000989~transcription factor activity, transcription factor binding	3	18.75	0.077915	9612, 144455, 3400	13	606	15478	5.894135567	0.9999737	0.394775	61.1185
GOTERM_MF_FAT	GO:0000988~transcription factor activity, protein binding	3	18.75	0.079262	9612, 144455, 3400	13	612	15478	5.836349925	0.9999782	0.386131	61.7752
GOTERM_MF_FAT	GO:0042802~identical protein binding	4	25	0.081447	144455, 29128, 2113, 604	13	1358	15478	3.50696726	0.999984	0.381329	62.818
GOTERM_BP_FAT	GO:0008285~negative regulation of cell proliferation	3	18.75	0.114615	144455, 2113, 604	16	651	16650	4.795506912	1	0.716711	85.7854
GOTERM_BP_FAT	GO:0030097~hemopoiesis	3	18.75	0.141223	2113, 604, 5452	16	739	16650	4.224458728	1	0.772686	91.2831
GOTERM_CC_FAT	GO:0044427~chromosomal part	3	18.75	0.187784	9612, 29128, 604	15	827	14527	3.513180169	0.9999962	0.998049	87.5618

Annotation Cluster 3 Enrichment Score: 1.6660094052876206

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0051726~regulation of cell cycle	5	31.25	0.00952	57472, 144455, 2113, 10018, 900	16	976	16650	5.331070697	0.9999775	0.291975	14.2122
GOTERM_BP_FAT	GO:0022402~cell cycle process	5	31.25	0.028959	57472, 144455, 3400, 10018, 900	16	1357	16650	3.834285188	1	0.432747	37.5587
GOTERM_BP_FAT	GO:0045787~positive regulation of cell cycle	3	18.75	0.03644	57472, 144455, 10018	16	339	16650	9.209070796	1	0.438368	44.8376

Annotation Cluster 4 Enrichment Score: 1.6319219610467002

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0033554~cellular response to stress	7	43.75	0.003092	57472, 144455, 29128, 2113, 5911, 10018, 604	16	1773	16650	4.108502538	0.9687499	0.319605	4.84237
UP_KEYWORDS	Ubl conjugation	6	37.5	0.005752	29128, 2113, 5911, 10018, 604, 6426	16	1705	20581	4.526612903	0.3474536	0.081832	5.85911
GOTERM_BP_FAT	GO:0032268~regulation of cellular protein metabolic process	7	43.75	0.012444	9612, 57472, 29128, 5911, 10018, 900, 604	16	2346	16650	3.105019182	0.9999992	0.329911	18.1824
GOTERM_BP_FAT	GO:0051246~regulation of protein metabolic process	7	43.75	0.017228	9612, 57472, 29128, 5911, 10018, 900, 604	16	2512	16650	2.899830812	1	0.357222	24.3083
GOTERM_BP_FAT	GO:0032270~positive regulation of cellular protein metabolic process	5	31.25	0.033552	9612, 29128, 5911, 10018, 604	16	1420	16650	3.664172535	1	0.429709	42.1281
GOTERM_BP_FAT	GO:0051247~positive regulation of protein metabolic process	5	31.25	0.040676	9612, 29128, 5911, 10018, 604	16	1508	16650	3.450348143	1	0.46631	48.5982
GOTERM_BP_FAT	GO:0051128~regulation of cellular component organization	6	37.5	0.047157	57472, 29128, 2113, 5911, 10018, 604	16	2323	16650	2.687795954	1	0.491185	53.8902
GOTERM_BP_FAT	GO:0033043~regulation of organelle organization	4	25	0.08052	57472, 29128, 10018, 604	16	1152	16650	3.61328125	1	0.616545	73.9547
GOTERM_BP_FAT	GO:0080134~regulation of response to stress	4	25	0.103968	2113, 5911, 10018, 604	16	1285	16650	3.239299611	1	0.686167	82.7839

Annotation Cluster 5 Enrichment Score: 1.5374758968271647

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0007049~cell cycle	7	43.75	0.002286	57472, 144455, 3400, 29128, 2113, 10018, 900	16	1672	16650	4.356683612	0.9227906	0.347453	3.60168
GOTERM_BP_FAT	GO:0000082~G1/S transition of mitotic cell cycle	3	18.75	0.01686	57472, 144455, 3400	16	224	16650	13.93694196	1	0.357565	23.8527
GOTERM_BP_FAT	GO:0044843~cell cycle G1/S phase transition	3	18.75	0.019806	57472, 144455, 3400	16	244	16650	12.79456967	1	0.378913	27.4285
GOTERM_BP_FAT	GO:0022402~cell cycle process	5	31.25	0.028959	57472, 144455, 3400, 10018, 900	16	1357	16650	3.834285188	1	0.432747	37.5587
GOTERM_BP_FAT	GO:1903047~mitotic cell cycle process	4	25	0.042792	57472, 144455, 3400, 900	16	890	16650	4.676966292	1	0.474781	50.3861
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	5	31.25	0.044551	57472, 144455, 3400, 2113, 604	16	1552	16650	3.352528995	1	0.479938	51.8268
GOTERM_BP_FAT	GO:0000278~mitotic cell cycle	4	25	0.052783	57472, 144455, 3400, 900	16	968	16650	4.300103306	1	0.502155	58.0646
GOTERM_BP_FAT	GO:0044772~mitotic cell cycle phase transition	3	18.75	0.076623	57472, 144455, 3400	16	514	16650	6.07368677	1	0.605135	72.1284
GOTERM_BP_FAT	GO:0044770~cell cycle phase transition	3	18.75	0.085328	57472, 144455, 3400	16	547	16650	5.70726691	1	0.635095	76.0539

Annotation Cluster 6 Enrichment Score: 1.494822164695718

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0044702~single organism reproductive process	6	37.5	0.003514	9612, 144455, 3400, 2113, 10018, 604	16	1231	16650	5.072095857	0.9805354	0.325589	5.48538
GOTERM_BP_FAT	GO:0002262~myeloid cell homeostasis	3	18.75	0.005422	2113, 10018, 604	16	124	16650	25.17641129	0.9977203	0.333411	8.34416
GOTERM_BP_FAT	GO:0022414~reproductive process	6	37.5	0.005635	9612, 144455, 3400, 2113, 10018, 604	16	1373	16650	4.547523671	0.9982057	0.326452	8.6579
GOTERM_BP_FAT	GO:0000003~reproduction	6	37.5	0.00567	9612, 144455, 3400, 2113, 10018, 604	16	1375	16650	4.540909091	0.9982755	0.312217	8.70978
GOTERM_BP_FAT	GO:0007160~cell-matrix adhesion	3	18.75	0.012585	54822, 10018, 604	16	192	16650	16.25976563	0.9999993	0.318197	18.369
GOTERM_BP_FAT	GO:0048872~homeostasis of number of cells	3	18.75	0.01686	2113, 10018, 604	16	224	16650	13.93694196	1	0.357565	23.8527
GOTERM_BP_FAT	GO:0031589~cell-substrate adhesion	3	18.75	0.030192	54822, 10018, 604	16	306	16650	10.20220588	1	0.424954	38.8174
GOTERM_BP_FAT	GO:0048534~hematopoietic or lymphoid organ development	4	25	0.030367	2113, 10018, 604, 5452	16	778	16650	5.350257069	1	0.421747	38.9949
GOTERM_BP_FAT	GO:0048609~multicellular organismal reproductive process	4	25	0.030873	9612, 2113, 10018, 604	16	783	16650	5.316091954	1	0.422074	39.503

GOTERM_BP_FAT	GO:0032504~multicellular organism reproduction	4	25	0.031795	9612, 2113, 10018, 604	16	792	16650	5.255681818	1	0.421799	40.4189
GOTERM_BP_FAT	GO:0002520~immune system development	4	25	0.034867	2113, 10018, 604, 5452	16	821	16650	5.070036541	1	0.437606	43.3774
GOTERM_BP_FAT	GO:0048646~anatomical structure formation involved in morphogenesis	4	25	0.080354	144455, 2113, 10018, 604	16	1151	16650	3.616420504	1	0.619525	73.8793
GOTERM_BP_FAT	GO:0080134~regulation of response to stress	4	25	0.103968	2113, 5911, 10018, 604	16	1285	16650	3.239299611	1	0.686167	82.7839
GOTERM_BP_FAT	GO:1902589~single-organism organelle organization	4	25	0.17185	54822, 5911, 10018, 604	16	1612	16650	2.58219603	1	0.830196	95.1291
GOTERM_BP_FAT	GO:0042592~homeostatic process	4	25	0.179378	54822, 2113, 10018, 604	16	1645	16650	2.530395137	1	0.836879	95.7922
GOTERM_BP_FAT	GO:0007155~cell adhesion	4	25	0.191919	54822, 2113, 10018, 604	16	1699	16650	2.449970571	1	0.856099	96.7125
GOTERM_BP_FAT	GO:0022610~biological adhesion	4	25	0.193329	54822, 2113, 10018, 604	16	1705	16650	2.441348974	1	0.856116	96.8032

Annotation Cluster 7 Enrichment Score: 1.4689816268702376

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0006974~cellular response to DNA damage stimulus	5	31.25	0.004869	57472, 144455, 29128, 10018, 604	16	807	16650	6.447490706	0.9957557	0.323049	7.52466
GOTERM_BP_FAT	GO:0033043~regulation of organelle organization	4	25	0.08052	57472, 29128, 10018, 604	16	1152	16650	3.61328125	1	0.616545	73.9547
GOTERM_BP_FAT	GO:0010638~positive regulation of organelle organization	3	18.75	0.099926	57472, 10018, 604	16	600	16650	5.203125	1	0.674359	81.4961

Annotation Cluster 8 Enrichment Score: 1.3685670029321517

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0033036~macromolecule localization	8	50	0.007163	375035, 3400, 5911, 10018, 345778, 604, 5452, 6426	16	2817	16650	2.955271565	0.9996791	0.284788	10.8819
GOTERM_BP_FAT	GO:0008104~protein localization	7	43.75	0.015249	375035, 3400, 5911, 10018, 345778, 604, 5452	16	2448	16650	2.975643382	1	0.356537	21.828
GOTERM_BP_FAT	GO:0045184~establishment of protein localization	6	37.5	0.02765	375035, 5911, 10018, 345778, 604, 5452	16	2021	16650	3.089435923	1	0.434745	36.1967
GOTERM_BP_FAT	GO:0034613~cellular protein localization	5	31.25	0.049019	3400, 5911, 10018, 345778, 604	16	1600	16650	3.251953125	1	0.492177	55.3133
GOTERM_BP_FAT	GO:0070727~cellular macromolecule localization	5	31.25	0.050273	3400, 5911, 10018, 345778, 604	16	1613	16650	3.225743955	1	0.496982	56.2481
GOTERM_BP_FAT	GO:0051641~cellular localization	6	37.5	0.065604	3400, 5911, 10018, 345778, 604, 6426	16	2541	16650	2.457201889	1	0.554148	66.2926
GOTERM_BP_FAT	GO:0072594~establishment of protein localization to organelle	3	18.75	0.119044	10018, 345778, 604	16	666	16650	4.6875	1	0.727795	86.8831
GOTERM_BP_FAT	GO:0033365~protein localization to organelle	3	18.75	0.193684	10018, 345778, 604	16	902	16650	3.461058758	1	0.854443	96.8257

Annotation Cluster 9 Enrichment Score: 1.2586418747632513

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0022402~cell cycle process	5	31.25	0.028959	57472, 144455, 3400, 10018, 900	16	1357	16650	3.834285188	1	0.432747	37.5587
GOTERM_BP_FAT	GO:0048608~reproductive structure development	3	18.75	0.053218	144455, 3400, 10018	16	418	16650	7.468600478	1	0.497207	58.3726
GOTERM_BP_FAT	GO:0061458~reproductive system development	3	18.75	0.05436	144455, 3400, 10018	16	423	16650	7.380319149	1	0.500897	59.17
GOTERM_BP_FAT	GO:0003006~developmental process involved in reproduction	3	18.75	0.110234	144455, 3400, 10018	16	636	16650	4.908608491	1	0.705198	84.6152

Annotation Cluster 10 Enrichment Score: 1.2003260506629123

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0001701~in utero embryonic development	3	18.75	0.034881	144455, 10018, 6426	16	331	16650	9.431646526	1	0.433094	43.3906
GOTERM_BP_FAT	GO:0009790~embryo development	4	25	0.05091	7358, 144455, 10018, 6426	16	954	16650	4.363207547	1	0.493321	56.716
GOTERM_BP_FAT	GO:0043009~chordate embryonic development	3	18.75	0.093502	144455, 10018, 6426	16	577	16650	5.410528596	1	0.659366	79.2625
GOTERM_BP_FAT	GO:0009792~embryo development ending in birth or egg hatching	3	18.75	0.095165	144455, 10018, 6426	16	583	16650	5.354845626	1	0.65904	79.8638

Annotation Cluster 11 Enrichment Score: 1.0417383981115396

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0007417~central nervous system development	4	25	0.044506	3400, 2113, 10018, 6426	16	904	16650	4.604535398	1	0.48398	51.7902
GOTERM_BP_FAT	GO:0007420~brain development	3	18.75	0.12442	3400, 2113, 10018	16	684	16650	4.564144737	1	0.73486	88.1087
GOTERM_BP_FAT	GO:0060322~head development	3	18.75	0.135357	3400, 2113, 10018	16	720	16650	4.3359375	1	0.763127	90.2783

Annotation Cluster 12 Enrichment Score: 1.0176444354141418

Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0007399~nervous system development	6	37.5	0.036429	3400, 2113, 5911, 10018, 604, 6426	16	2170	16650	2.877304147	1	0.442814	44.8277
GOTERM_BP_FAT	GO:0008284~positive regulation of cell proliferation	4	25	0.037979	57472, 3400, 2113, 604	16	849	16650	4.902826855	1	0.447619	46.2333
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	5	31.25	0.044551	57472, 144455, 3400, 2113, 604	16	1552	16650	3.352528995	1	0.479938	51.8268
GOTERM_BP_FAT	GO:0034613~cellular protein localization	5	31.25	0.049019	3400, 5911, 10018, 345778, 604	16	1600	16650	3.251953125	1	0.492177	55.3133
GOTERM_BP_FAT	GO:0070727~cellular macromolecule localization	5	31.25	0.050273	3400, 5911, 10018, 345778, 604	16	1613	16650	3.225743955	1	0.496982	56.2481
GOTERM_BP_FAT	GO:0045664~regulation of neuron differentiation	3	18.75	0.088841	3400, 5911, 604	16	560	16650	5.574776786	1	0.643274	77.4859
GOTERM_BP_FAT	GO:0080134~regulation of response to stress	4	25	0.103968	2113, 5911, 10018, 604	16	1285	16650	3.239299611	1	0.686167	82.7839
GOTERM_BP_FAT	GO:0050767~regulation of neurogenesis	3	18.75	0.123819	3400, 5911, 604	16	682	16650	4.577529326	1	0.736196	87.9773
GOTERM_BP_FAT	GO:0022008~neurogenesis	4	25	0.135951	3400, 5911, 604, 6426	16	1447	16650	2.876641327	1	0.761728	90.3848
GOTERM_BP_FAT	GO:0051960~regulation of nervous system development	3	18.75	0.150607	3400, 5911, 604	16	769	16650	4.059655397	1	0.792917	92.6904
GOTERM_BP_FAT	GO:0045595~regulation of cell differentiation	4	25	0.156688	3400, 2113, 5911, 604	16	1544	16650	2.695919689	1	0.804051	93.4855
GOTERM_BP_FAT	GO:0051270~regulation of cellular component movement	3	18.75	0.161086	2113, 5911, 604	16	802	16650	3.892612219	1	0.810936	94.0091
GOTERM_BP_FAT	GO:0045597~positive regulation of cell differentiation	3	18.75	0.173661	3400, 2113, 604	16	841	16650	3.712098692	1	0.83115	95.297
GOTERM_BP_FAT	GO:0060284~regulation of cell development	3	18.75	0.176916	3400, 5911, 604	16	851	16650	3.668478261	1	0.834791	95.5853
GOTERM_BP_FAT	GO:2000026~regulation of multicellular organismal development	4	25	0.197103	3400, 2113, 5911, 604	16	1721	16650	2.418651947	1	0.855471	97.0347

Annotation Cluster 13	Enrichment Score: 0.9681395697517											
Category	**Term**	**Count**	**%**	**PValue**	**Genes**	**List Total**	**Pop Hits**	**Pop Total**	**Fold Enrichment**	**Bonferroni**	**Benjamini**	**FDR**
GOTERM_MF_FAT	GO:0000166~nucleotide binding	5	31.25	0.100225	7358, 29128, 54822, 5911, 6426	13	2389	15478	2.491869788	0.9999989	0.435637	70.7675
GOTERM_MF_FAT	GO:1901265~nucleoside phosphate binding	5	31.25	0.100348	7358, 29128, 54822, 5911, 6426	13	2390	15478	2.490827164	0.9999989	0.422985	70.814
GOTERM_MF_FAT	GO:0036094~small molecule binding	5	31.25	0.123907	7358, 29128, 54822, 5911, 6426	13	2571	15478	2.315471382	1	0.483879	78.5725

Annotation Cluster 14	Enrichment Score: 0.914480077900074											
Category	**Term**	**Count**	**%**	**PValue**	**Genes**	**List Total**	**Pop Hits**	**Pop Total**	**Fold Enrichment**	**Bonferroni**	**Benjamini**	**FDR**
GOTERM_BP_FAT	GO:0030036~actin cytoskeleton organization	3	18.75	0.086944	54822, 5911, 604	16	553	16650	5.64534358	1	0.638618	76.7228
GOTERM_BP_FAT	GO:0030029~actin filament-based process	3	18.75	0.120829	54822, 5911, 604	16	672	16650	4.645647321	1	0.73018	87.3025
GOTERM_BP_FAT	GO:1902589~single-organism organelle organization	4	25	0.17185	54822, 5911, 10018, 604	16	1612	16650	2.58219603	1	0.830196	95.1291

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